J. Membrane Biol. 1, 79–91 (1969)

The Cellular Specificity of the Effect of Vasopressin on Toad Urinary Bladder

DONALD R. DIBONA, MORTIMER M. CIVAN, and ALEXANDER LEAF

Departments of Medicine, The Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts 02114

Received 11 March 1969

Summary. Phase and electron micrographs of toad bladders were obtained following dilution of bathing media in the presence and absence of vasopressin. Dilution of the mucosal medium alone resulted in no morphologic changes. Subsequent addition of vasopressin produced an increase in the cell volume of the granular cells, manifested by some or all of the following changes: increased area of granular cell profiles as observed in sections, rounding of the cell nucleus, displacement of the two components of the nuclear envelope, loss of nuclear heterochromatin, sacculation of the endoplasmic reticulum and the Golgi apparatus, and reduction in the electron density of the cell cytoplasm. No such morphologic changes were noted in the other cell types comprising the mucosal epithelium – the mitochondria-rich, the goblet, and the basal cells. On the other hand, dilution of the serosal bathing medium in the absence of vasopressin caused a marked increase in the cell volume of all these cell types. The results demonstrate that the action of vasopressin to enhance bulk water flow across toad bladder is exerted specifically on the apical surface of the granular cells. It is suggested that the hormonal effect on sodium transport may also be limited to the granular cells. The route of osmotic water flow and the possible role of the other mucosal epithelial cells is discussed.

The hormone vasopressin causes an increase both in osmotic water flow and in net sodium transport across the urinary bladder of the toad [7]. Several lines of evidence indicate that both effects are mediated at the apical membranes of the mucosal cells:

1) Studies with ^{24}Na, ^{14}C-labeled urea, and tritiated water applied to the mucosal medium show an increase in each instance of radioactivity within the tissue in response to vasopressin [5, 7].

2) Reduction of the tonicity of the mucosal medium does not alter cell morphology until vasopressin is added, after which the mucosal cells are markedly swollen [9, 1].

3) Determinations of the resistance profile with glass micropipettes have demonstrated that the fall in tissue resistance induced by vasopressin occurs across an apical permeability barrier [3]; since the mucosal epithelium consists of a single complete layer of cells [4], this barrier is likely to be the apical plasma membrane of the mucosal cells.

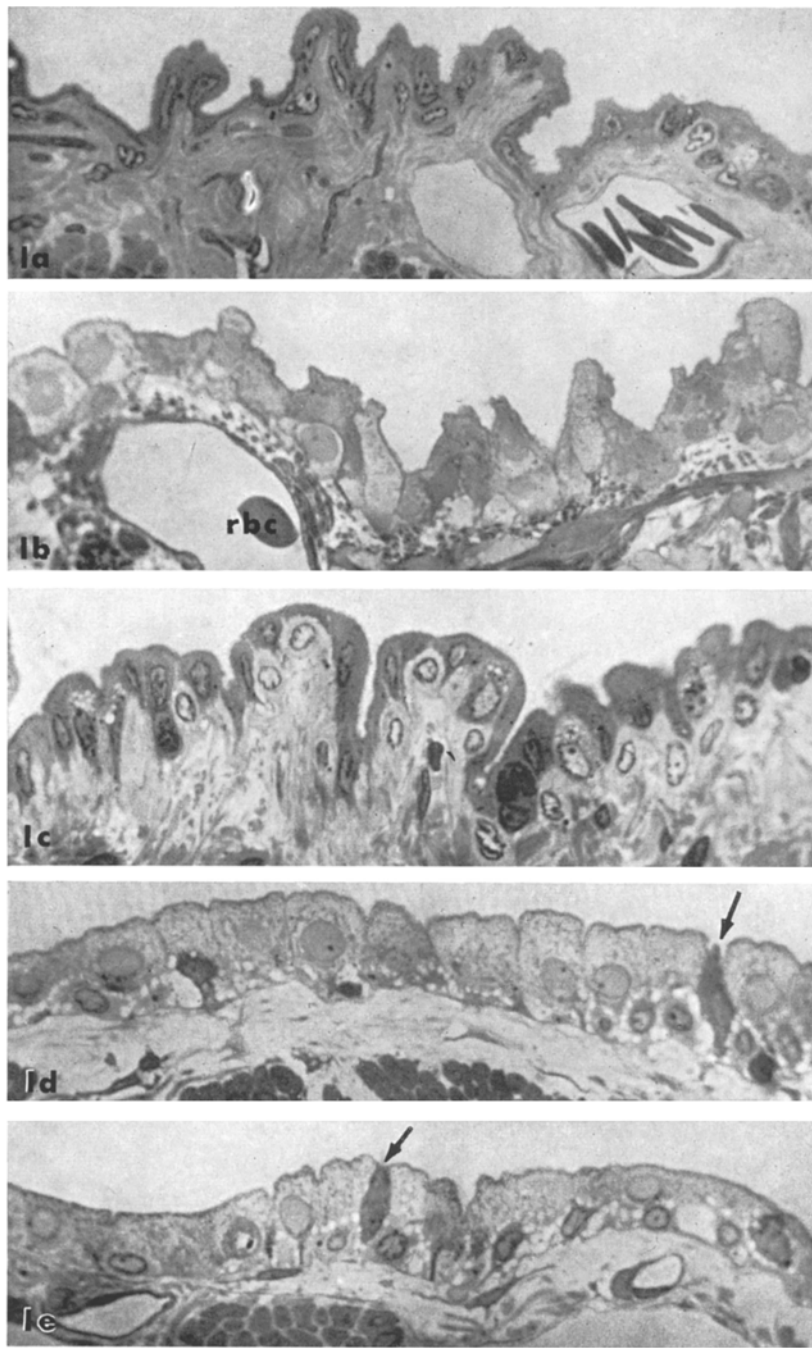

Fig. 1a – e. Phase micrographs of toad urinary bladder. a) Control quarter-bladder – bathed – with isotonic (220 mosm) Ringer's solution on mucosal and serosal surfaces. Fig. 1a and b are from the same double-chamber experiment. × 600. b) Experimental quarter-bladder – bathed with isotonic (220 mosm) Ringer's solution on mucosa and hypotonic (110 mosm)

Although these observations indicate an apical site of hormonal action, there are four cell types (granular cells, mitochondria-rich cells, goblet cells and basal cells; [2] comprising the mucosal epithelium, the first three of which contact the urinary surface [4]. To investigate the possibility that one of these cell types constitutes a specific cell receptor for vasopressin, the present studies were performed. The results suggest that the granular cells are specifically responsive to vasopressin and that the route of osmotic water flow is through the granular cells rather than through the apical tight junctions.

Methods

Female specimens of the toad, *Bufo marinus*, (obtained from the Dominican Republic, National Reagents Inc., Bridgeport, Conn.) were maintained on moist earth at room temperature after forced feeding of mealworms upon arrival. Urinary hemibladders were excised from doubly pithed toads, rinsed in Ringer's solution, and mounted in a Lucite double-chamber, which permitted measurement of net water flow, electrical potential, and short-circuit current across the preparation [8]. The tissues were bathed either with chloride or sulfate Ringer's solutions. The chloride Ringer's solution contained: Na^+, 113; K^+, 3.5; Ca^{++}, 0.9; Cl^-, 116; HCO_3^-, 2.4 mM; pH, 7.5 to 8.0; and tonicity, 220 mosm/kg H_2O. In several experiments, the Ringer's solution was further buffered with $H_2PO_4^-$, 0.3; HPO_4^{--}, 1.5 mM. The sulfate Ringer's solution was of the following composition: Na^+, 113; K^+, 3.7; Ca^{++}, 0.9; SO_4^{--}, 57; HPO_4^{--}, 1.7; $H_2PO_4^-$, 0.3; mannitol, 58 mM; pH, 7.3; and tonicity, 210 mosm/kg H_2O. In most of the experiments performed, both net volume flow and short-circuit current were monitored throughout the experiment by techniques previously described [8]. Either vasopressin (Pitressin®, Parke, Davis and Company, Detroit, Mich.) was added to a final serosal concentration of 167 to 200 m.u./ml or vasotocin (Sandoz S. A., Basel, Switzerland) was added to a final serosal concentration of 0.2 to 1.1×10^{-5} mg/ml; 19 to 58 min after this, the tissues were fixed with 1% glutaraldehyde (Fisher Scientific Company, Pittsburgh, Pa.). The preparations were subsequently postfixed with osmium, and then dehydrated, sectioned, and embedded as described previously [4]. Sections were stained with uranyl acetate and lead citrate and examined in a Philips EM-200 electron microscope. Phase microscopy was done on 1.0- to 1.5-μ sections with Zeiss optics.

Results

Phase microscopic findings are summarized in Fig. 1. It is clear that a dilute solution on the mucosal surface results in a swollen epithelium

solution on serosa. Note the apparent increase in epithelial cell size and in submucosal elements, e.g., red blood cells (rbc), over the control sample shown above (Fig. 1a). × 600. c) Control quarter-bladder – bathed in isotonic (210 mosm) Ringer's solution on serosa, hypotonic (105 mosm) solution on mucosa. Dilution of the mucosal medium has not changed the appearance of the epithelium from that shown in Fig. 1a where all solutions were isotonic. Fig. 1c, d, and e are from the same double-chamber experiment. × 600. d) and e) Experimental quarter-bladder – bathed with an isotonic (210 mosm) serosal solution and hypotonic (105 mosm) mucosal solution, as above in Fig. 1c, but with addition of vasopressin to the serosal medium. Cell swelling in the epithelium is evident, but note that there are some cells (arrows) that are appreciably more dense than the rest. × 600

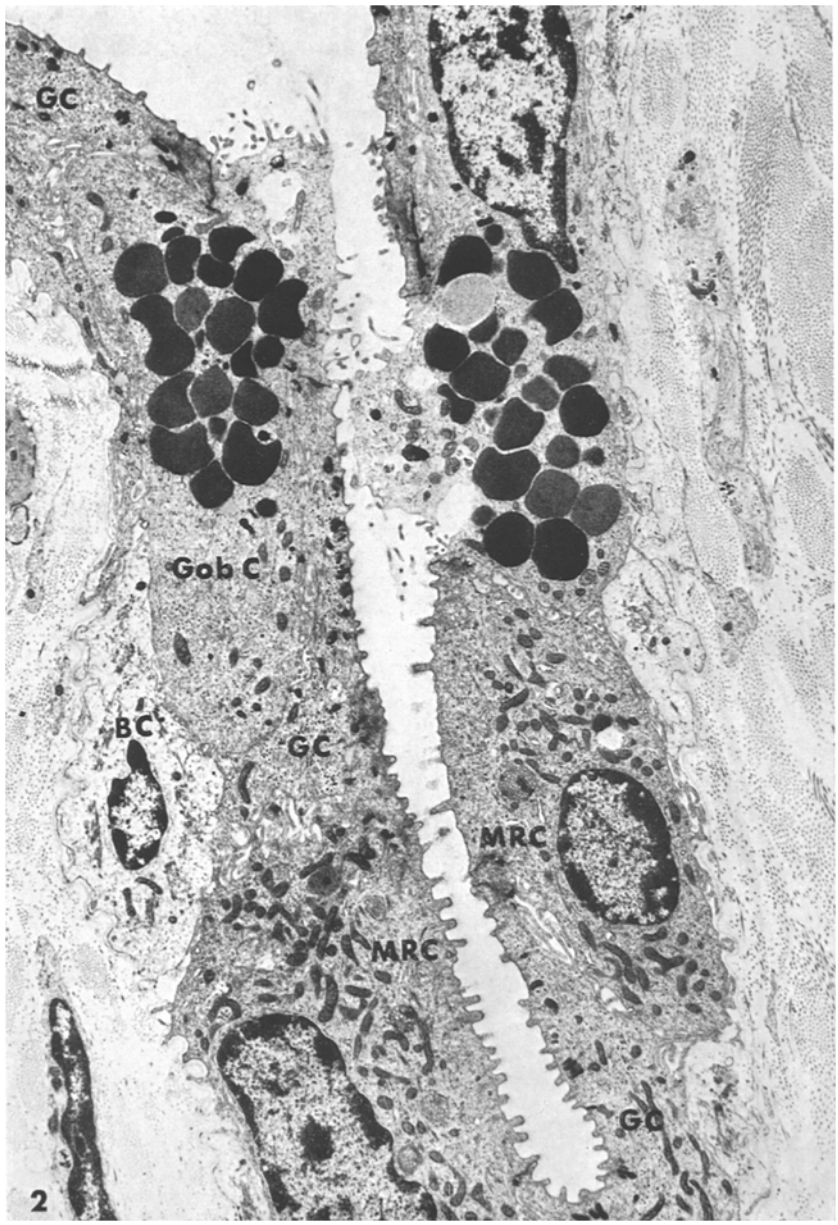

Fig. 2. Electron micrograph of preparation shown by phase microscopy in Fig. 1a. Survey view of toad bladder epithelium fixed after both surfaces have been bathed in isotonic (220 mosm) Ringer's solution. Granular cells (*GC*), goblet cells (*Gob c*), mitochondria-rich cells (*MRC*), and basal cells (*BC*) are all present in this field. Note that basal cells (*BC*) show considerably less cytoplasmic density than the three remaining cell types which are comparable to one another in this respect. × 6,000

only in the presence of vasopressin. This well-established result [9, 1] is, however, necessarily modified for there are, in Fig. 1d and e, some epithelial cells which have an obviously greater density than do the majority of cells facing the mucosal surface. These photographs are supported by numerous phase microscopic observations on similarly treated tissue where a small percentage of mucosal cells appear unaffected by mucosal hypotonicity, even in the presence of vasopressin or vasotocin. The "darker" cells might well be mitochondria-rich or goblet cells, but a nonambiguous determination of cell type is not truly possible with phase microscopy.

On the other hand, in tissues exposed to hypotonic serosal media (Fig. 1b), the effect on the various cells is considerably more uniform than in the case of vasopressin-induced water flow (Fig. 1d, e).

In order to establish the precise identity of the cells which remain unaffected by vasopressin, electron micrographs were obtained. Representative examples of the four histologic types [2] from a preparation bathed in isotonic media are presented in Fig. 2. The granular, mitochondria-rich, and goblet cells have been demonstrated to contact both urinary surface and basement membrane, whereas the basal cells constitute an incomplete second layer interposed between surface cells and basement membrane [4].

Granular cells from tissues bathed in isotonic Ringer's solution contained a cytoplasm consistently denser than that of the basal cells but very similar in density to that of the mitochondria-rich cells. The nuclei of each of the cell types in the reference state were irregularly lobed and contained appreciable clumps of heterochromatin.

Electron micrographs of tissue exposed to hypotonic mucosal media (Figs. 1c, 3a) were indistinguishable from those bathed only in isotonic solutions. The addition of vasopressin or vasotocin, however, resulted in some or all of a number of striking changes in the granular cells. Figure 3b, c, and d demonstrates the appearance of the epithelium fixed during vasopressin-induced osmotic water movement. Sectioned regions generally show much larger profiles for granular cells. These cells, furthermore, have lost much of the background cytoplasmic density of the reference state. Nuclei are rounded; virtually all of the heterochromatin has disappeared in most cases; and the rough-surfaced outer membrane of the nuclear envelope is occasionally distended. Smooth-surfaced circular membrane profiles are found in the supranuclear region where the cytoplasm is frequently far less dense than in the basal aspect of these cells. A Golgi apparatus, although seen in the characteristic form of flattened

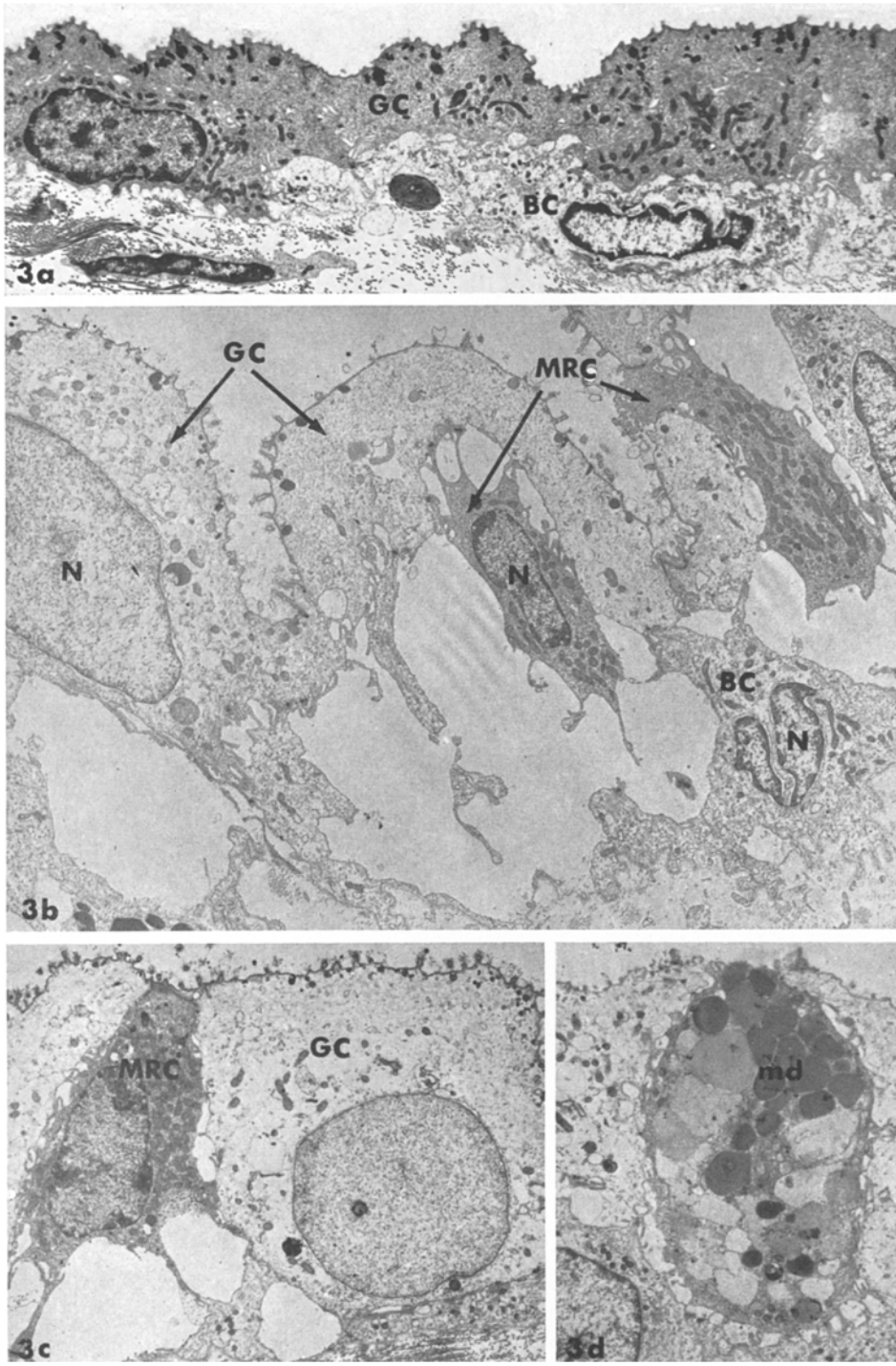

Fig. 3a – c

saccules and small vesicles under isotonic conditions is never found as such in these cells; instead, one or more large vesicles are frequently observed on the apical side of the nucleus.

Although the granular cells showed these signs of apparent swelling, mitochondria-rich cells (*see* Figs. 3b, c, 4c) did not show any of the features noted above. Mitochondria-rich cells form a minority population comprising 8 to 10% of the cells reaching the mucosal margin [2]. However, 53 such cells were recorded. None of these showed signs of swelling while each was in contiguity with obviously affected granular cells on either side. Basal cells also seemed unaffected. The shift in contrast between basal and granular cell cytoplasms is most clear in a comparison of Fig. 4a with Fig. 4b and c.

Figure 4 is provided to offer an experiment with control in the following sense: both halves have been treated with vasopressin, the mucosal medium was hypotonic in one half (Fig. 4b, c) and isotonic on the other (Fig. 4a). It is of interest that the apparent enlargement of intercellular spaces occurs both in the presence and absence of water movement; this is the subject of another study.

The details of swelling and the presence of a gradient of density within individual granular cells is detailed in Fig. 5.

To distinguish between the possibility of preservation artifacts and a true differential response to vasopressin by the various cell types, bladders were bathed with hypotonic serosal media. The four epithelial cell types are depicted in Fig. 6; all types show evidences of a hypotonic environment, most noticeably the complete loss of heterochromatin by nuclei. The mitochondria-rich cells (Fig. 6a, b) are unmistakably much more affected than under conditions of vasopressin-induced water flow.

Fig. 3a–d. Electron micrographs of preparation shown by phase microscopy in Fig. 1c, d, and e. a) Low-power view of epithelium fixed as in Fig. 1c (dilute mucosa, isotonic serosa). Epithelial cells appear much the same as in Fig. 2; basal cells (*BC*) still show a far less dense cytoplasm than granular cells (*GC*). × 5,000. b) Bladder epithelium exposed to identical osmotic gradient as in Fig. 3a, but with addition of vasopressin to serosal medium. Note the marked distension of the granular cells (*GC*) as compared to the mitochondria-rich cells (*MRC*). Basal cells (*BC*) show a cytoplasmic density slightly greater than that of the granular cells, as opposed to the situation in Fig. 3a. The intercellular spaces are strikingly enlarged. Granular cell nuclei (*N*) show little, if any, heterochromatin while those of other cells retain this feature and still show an irregular lobulation. × 5,000. c) Example of the striking differences in size and appearance of granular cell (*GC*) and mitochondria-rich cell (*MRC*) nuclei fixed during vasopressin-induced water flow. Notice also the vesiculation (*v*) of granular cell cytoplasm. × 5,000. d) Goblet cell of bladder epithelium fixed during osmotic water flow. While the mucin droplets (*md*) here are somewhat less dense than those in Fig. 2, such variation is not uncommon. This cell is included to show that it has not suffered marked distension. × 5,000

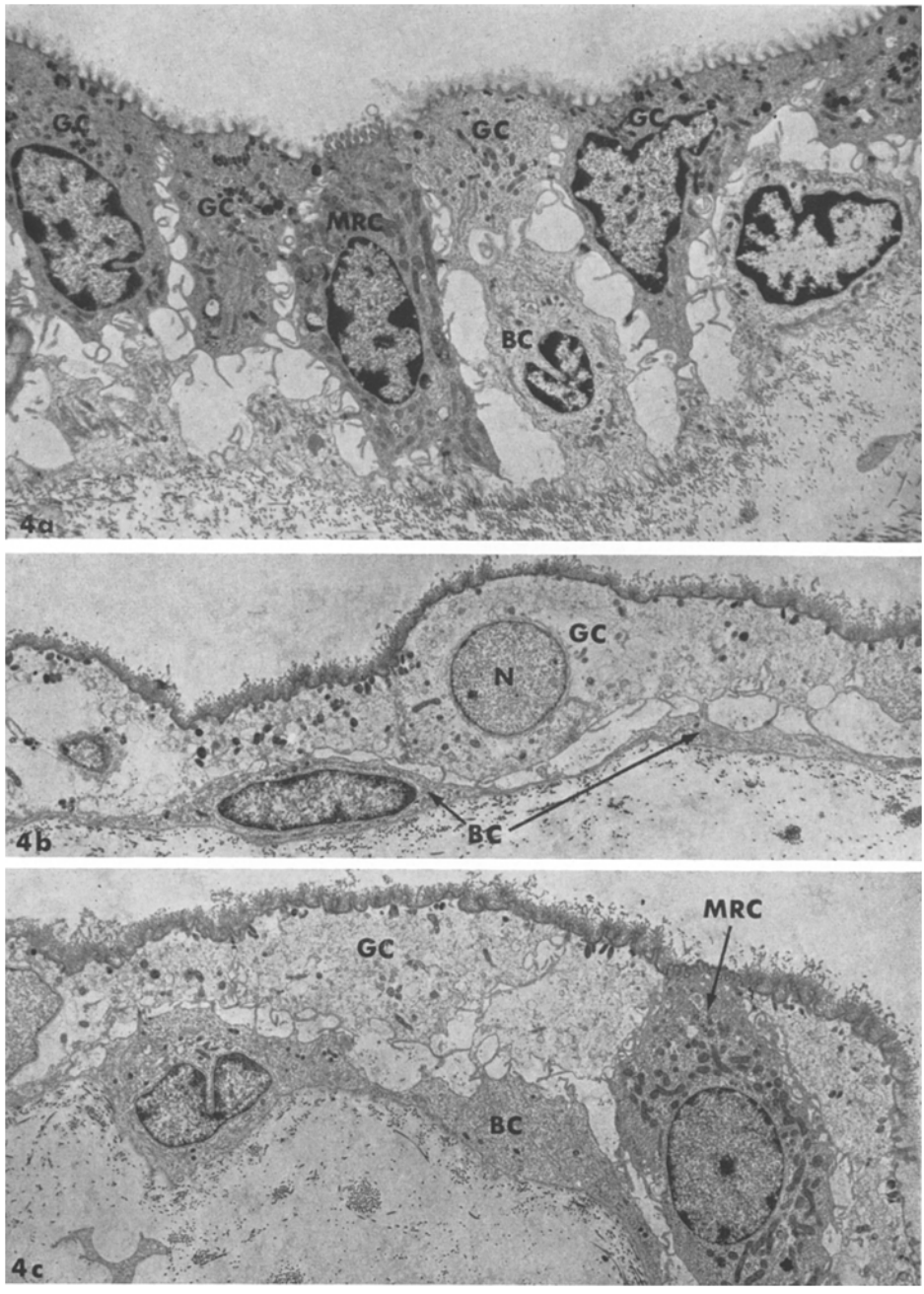

Fig. 4a–c. Electron micrographs from an experiment designed to show the effect of vasopressin in the presence (Fig. 4b, c) and absence (Fig. 4a) of an osmotic gradient. a) Low-power view of epithelium showing the similarity in cytoplasmic appearance of mitochondria-rich (*MRC*) and granular cells (*GC*). The intercellular spaces are enlarged in the presence of vasopressin despite the absence of transepithelial gradient. × 5,000. b) Epithelium fixed during

Discussion

The results above, based upon phase and electron micrographs, indicate a change in cell volume accompanying vasopressin-induced water flow. Phase microscopy has been of considerable value in that sampling of large cell populations is possible, but difficulty is encountered when one tries to establish the precise identity of a given cell. On the other hand, while electron microscopy permits cell identification, the sampling is very small and a rigidly quantitative approach to cell volume would have required extensive serial sectioning.

This problem was resolved by employing two complementary techniques. First, each preparation consisted of an experimental and a control half subjected to the same fixative and preparative procedures; large changes in cell volume could then be appreciated by visually comparing the cell outlines of a given histologic type sectioned from the experimental and control halves of the tissue. Second, the size, shape, density, and fine structure of the cellular organelles, as well as the density of the cytoplasm, constituted a set of internal standards permitting assessment of cellular response.

The present studies confirm previous reports [9, 1] that a reduction in the tonicity of the mucosal bathing fluid results in little or no change in morphology of the toad bladder. However, in the presence of a hypotonic mucosal medium, vasopressin induced an apparent increase in cell area, a reduction in the electron density of the cell cytoplasm, a rounding of the nuclear outline, occasional displacement of the two components of the nuclear envelope, a loss of nuclear heterochromatin, a sacculation of the endoplasmic reticulum and Golgi apparatus of the granular cells alone; no such changes are induced in the mitochondria-rich, goblet, or basal cells. It is concluded that in the presence of a hypotonic mucosal medium, vasopressin selectively increases the volume of the granular cells of the mucosal epithelium.

Reduction of the tonicity of the serosal bathing medium, however, results in a marked increase in cell volume of each of the four cell types, assessed on the basis of the above criteria. The increase in cell volume solely of the granular cells following vasopressin must, therefore, reflect a

water flow from mucosa to serosa. Granular cell nucleus (*N*) is rounded considerably, and the contrast in granular (*GC*) and basal cell (*BC*) cytoplasm is reversed with regard to the reference state (Fig. 4a). × 5,000. c) View of epithelium showing the appearance of a mitochondria-rich cell (*MRC*) fixed during osmotic water movement. No marked changes are evident when this cell is compared to that in Fig. 4a. × 5,000

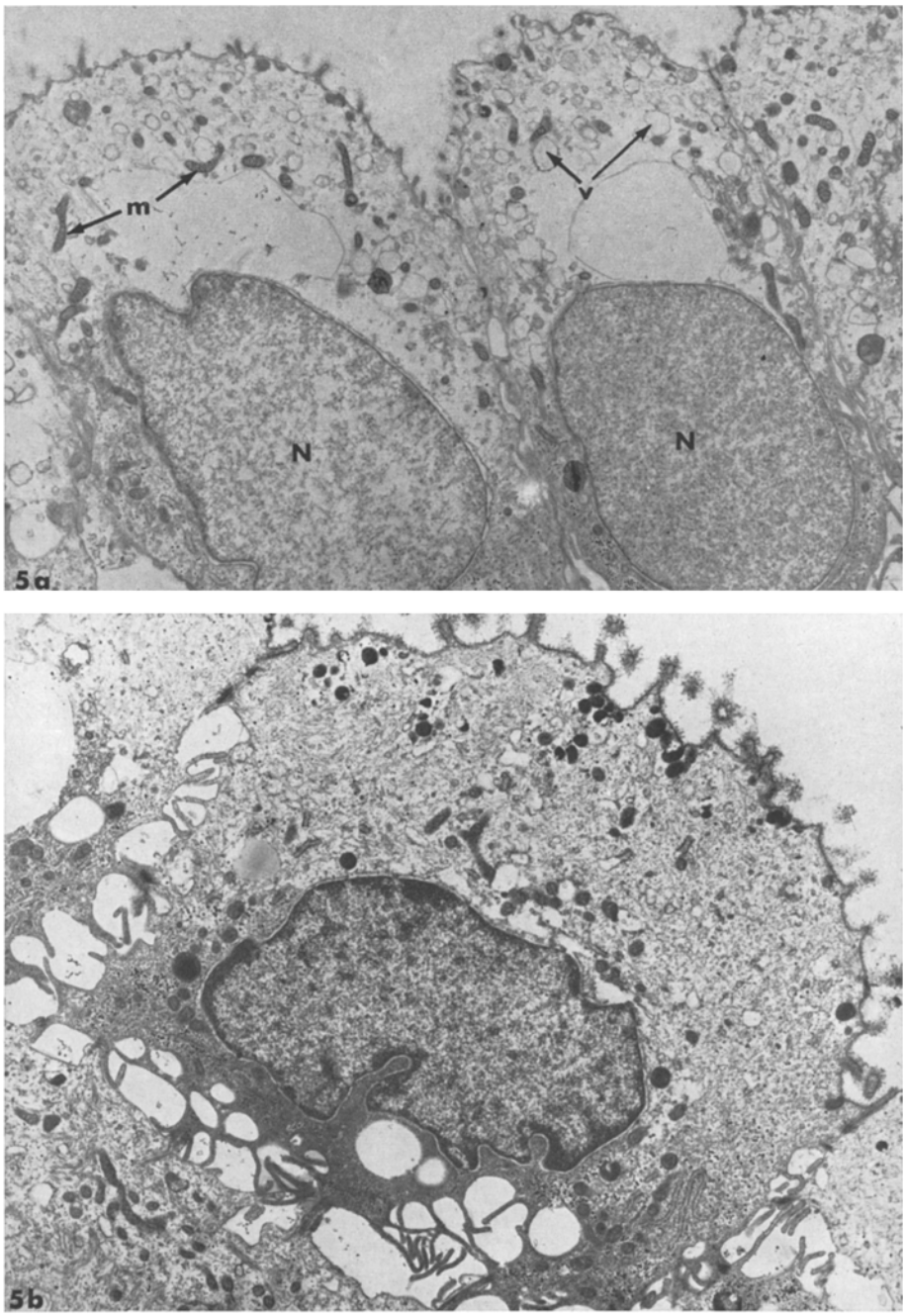

Fig. 5a and b. Electron micrographs detailing the appearance of granular cells during vasopressin-induced water flow. a) Two granular cells with a high degree of vesiculation (*v*) in the apical cytoplasm. Note that the cytoplasm grows more dense in the regions surrounding the nucleus (*N*). The mitochondria (*m*) do not seem affected. × 10,500. b) Granular cell somewhat less affected by osmotic water movement in that the nucleus has retained much of the heterochromatin of the reference condition. This field has been reproduced with an increase in contrast to emphasize the gradient of cytoplasmic density found within this cell. × 10,500

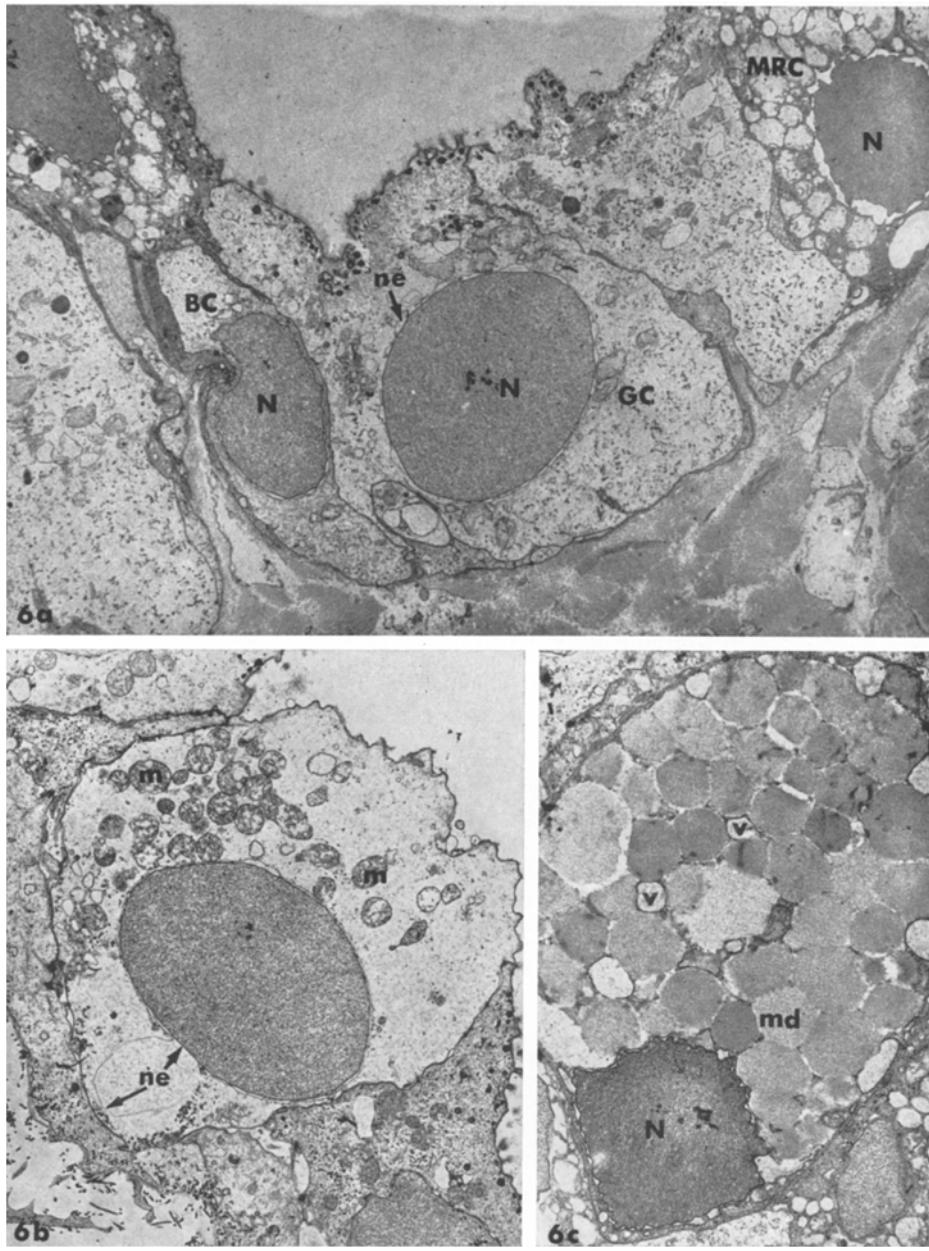

Fig. 6a–c. Electron micrographs of preparation shown by phase microscopy in Fig. 1b. a) Survey view of epithelium fixed after dilution of serosal bathing medium. Basal cells (*BC*), mitochondria-rich cells (*MRC*), and granular cells (*GC*) are all affected by this procedure. Note that heterochromatin is absent from all cell nuclei (*N*) and that nuclear envelopes (*ne*) become very apparent as the two-component system is separated. × 5,000. b) Mitochondria-rich cell with marked changes following exposure to hypotonicity on basal and lateral surfaces. The nuclear envelope (*ne*) is dramatically distorted; several mitochondria (*m*) are disrupted. × 7,000. c) Goblet cell showing a nucleus (*N*) without heterochromatin, with distorted nuclear envelope (*ne*), and with cytoplasmic vesiculation (*v*) in available space between mucin droplets (*md*). × 7,000

hormonal specificity rather than a nonspecific limitation in the capacity of the other cell types to swell.

The observation of cell swelling induced by bulk water flow from mucosal-to-serosal surfaces in the presence of vasopressin and a dilute mucosal medium is consistent with the current concept that water flow occurs across the cells rather than only between them. The presence of a density gradient of the intercellular constituents, compactness of cytoplasm being the least at the apical surface and the greatest near the basal surface of the granular cells, is consistent with movement of water across the apical plasma membrane and then through the cell across the lateral and basal surfaces. A similar appearance might result from water moving only through the apical "tight" intercellular junctions and then into the cells across their lateral surfaces. This, however, seems unlikely as the mitochondria-rich cells would then be expected to participate in such swelling, since these cells, like the granular cells, have been shown to swell when dilute medium is applied to their basal and lateral surfaces.

Since both radioactive tracer [5] and electrophysiologic [3] data indicate that the effect of vasopressin on sodium transport is mediated at the apical plasma membrane, and since the apical effect of vasopressin on bulk water flow is limited to the granular cells, it is reasonable to assume that the hormonal effect on net sodium transport is also limited to the granular cells. If so, a question arises as to the function of the other three cell types. The goblet cells secrete mucus in a direction opposite to that of net water and salt movement under physiological conditions. The basal cells do not contact the urinary surface and do not constitute by themselves a complete cell layer [4]; Choi [2] has suggested that they are germinal cells which later develop into granular or mitochondria-rich cells.

The mitochondria-rich cells contain a higher concentration of mitochondria than do the granular cells, suggesting that they subserve some function coupled to oxidative metabolism. It is of interest that the hormone aldosterone stimulates net sodium transport across toad bladder and that this action is largely dependent upon the presence of oxygen [10]. Furthermore, the increments in net sodium transport which follow addition of aldosterone and vasopressin are additive in freshly mounted tissues [10]. These data permit the speculation that the mitochondria-rich cells are specifically responsive to the mineralocorticoid effect of aldosterone; speculation with regard to specificity of the glucocorticoid effect of aldosterone is not similarly warranted [6].

This study was supported in part by the John A. Hartford Foundation, Inc., and by U.S. Public Health Service grants HE-06664 from the National Heart Institute and AM-04501 from the National Institute of Arthritis and Metabolic Diseases.

References

1. Carasso, N., P. Favard, and J. Valérien. 1962. Variations des ultrastructures dans les cellules épithéliales de la vessie du crapaud après stimulation par l'hormone neurohypopophysaire. *J. Microscopie* **1**:143.
2. Choi, J. K. 1963. The fine structure of the urinary bladder of the toad, *Bufo marinus*. *J. Cell Biol.* **16**:53.
3. Civan, M. M., and H. S. Frazier. 1968. The site of the stimulatory action of vasopressin on sodium transport in toad bladder. *J. Gen. Physiol.* **51**:589.
4. DiBona, D. R., M. M. Civan, and A. Leaf. 1969. The anatomic site of the transepithelial permeability barriers of toad bladder. *J. Cell Biol.* **40**:1.
5. Frazier, H. S., E. F. Dempsey, and A. Leaf. 1962. Movement of sodium across the mucosal surface of the isolated toad bladder and its modification by vasopressin. *J. Gen. Physiol.* **45**:529.
6. Handler, J. S., A. S. Preston, and J. Orloff. 1968. Some effects of adrenal steroid hormones and the toad's urinary bladder. Am. Soc. Nephrol. (Abstract) 24.
7. Leaf, A. 1960. Some actions of neurohypophyseal hormones on a living membrane. *J. Gen. Physiol.* **43**:175.
8. Lichtenstein, N. S., and A. Leaf. 1965. Effect of amphotericin B on the permeability of the toad bladder. *J. Clin. Invest.* **44**:1328.
9. Peachey, L. D., and H. Rasmussen. 1961. Structure of the toad's urinary bladder as related to its physiology. *J. Biophys. Biochem. Cytol.* **10**:529.
10. Sharp, G. W. G., and A. Leaf. 1966. Mechanism of action of aldosterone. *Physiol. Rev.* **46**:593.