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Structural Responses to Voltage-Clamping in the Toad Urinary Bladder

II. Granular Cells and the Natriferic Action of Vasopressin

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Summary. The natriferic action of vasopressin has been investigated with morphological studies of voltage-clamped toad urinary bladders. Granular cell swelling can be induced in the presence of isoosmotic solutions when the orientation of the transmural potential is reversed by voltage clamping (V.A. Bobrycki, J.W. Mills, A.D.C. Macknight & D.R. DiBona, *J. Membrane Biol.*, **60**:21, 1981) and results from an increased rate of sodium entry across the mucosal membrane; under these conditions the active transport mechanism at the basal-lateral membrane becomes rate-limiting. Vasopressin exacerbated the voltage-reversal-induced swelling of granular cells while other cell types were unaffected. Granular cell swelling appeared to be dependent upon sodium entry from the mucosal medium since it was completely prevented by amiloride. There was no evidence for an effect of vasopressin on tight junction permeability; voltage-reversal induced the same amount of junction blistering whether or not vasopressin was present. It is concluded that the predominant effect of vasopressin on transepithelial sodium transport is to increase the sodium conductance of the mucosal plasma membrane. As is the case with the hydroosmotic effect of the hormone, the natriferic action of vasopressin seems to be exerted primarily, if not entirely, on the granular cells.

The results presented in the preceding paper (Bobrycki, Mills, Macknight, & DiBona, 1980) established that the imposition of a potential-reversing voltage clamp across toad urinary bladder could induce a swelling of granular cells which was specifically dependent on the amiloride-sensitive entry of sodium to the cells from the mucosal medium. Under these experimental conditions, it seems that the rate-limiting step in transepithelial sodium transport becomes the active transport step at the basal-lateral cell membrane rather than the passive entry process at the apical membrane. Thus sodium driven into the cell from the mucosal medium down the increased electrical gradient, and chloride gained from the serosal medium to maintain cellular electrical neutrality, accumulate within the cell and oblige an osmotic gain in cellular water.

The purpose of the present paper is to examine the effect of vasopressin on the volume of toad bladder epithelial cells. This hormone stimulates active sodium transport in this tissue (Leaf, 1965). Vasopressin is believed to act by increasing apical membrane permeability to sodium (Civan & Frazier, 1968; Macknight, Leaf & Civan, 1971) although an additional specific effect of the hormone directly on the active process at the basal-lateral membrane has been suggested (Finn, 1968; Janacek & Rybova, 1971; Aceves, 1977). Although its effect on the granular cells in promoting the osmotic flow of water across the tissues is well established (DiBona, Civan & Leaf, 1969), there is still controversy over the specific cellular type involved in the mediation of the increased transport of sodium which the hormone produces (Scott, Sapirstein & Yoder, 1974; Handler & Preston, 1976). These issues have been investigated in the present study using the voltage-clamping procedures described in the previous paper (Bobrycki et al., 1980).

The results we have obtained support the hypothesis that the major effect of vasopressin on sodium transport is to promote the entry of sodium to the cells via an amiloride-sensitive process and show that it is the granular cells, specifically, whose apical membrane sodium-permeability is affected by the hormone.

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Materials and Methods

Female toads, *Bufo marinus*, were obtained from the Dominican Republic through National Reagents Inc. (Bridgeport, Conn.) and were kept on moist wood chips without food for periods of up to three weeks before being sacrified. Animals were double-pithed and urinary hemibladders dissected free for mounting in Lucite double chambers. In this way control and experimental sheets of tissues (each 2.5 cm^2 in area) were obtained from adjacent portions of a single hemibladder for each experiment. Tissue was held flat in the chamber by application of about 1 cm hydrostatic pressure from the mucosal bath with the serosal aspect resting on a sheet of nylon mesh.

The sodium Ringer's solution consisted of (mM): Na^+, 117; K^+, 3.5; Ca^{2+}, 1.0; Cl^-, 117; HPO_4^{2-}, 2; the pH was 7.8 and osmolality was 228–235 mOsm/kg H_2O. Amiloride was a generous gift of Merck, Sharpe and Dohme (Rayway, N.J.) and was used at 10^{-4} M concentration in the mucosal bath. When used, vasopressin (Pitressin, Parke, Davis and Co., Detroit) was applied to the serosal bathing medium to provide a supramaximal effective concentration of 100 mU/ml.

Transmural potential difference was monitored with calomel electrodes; voltage-clamping was applied through chlorided silver electrodes; electrical continuity from electrode reservoirs to the bathing media was by means of 3 M KCl agar bridges. The electrophysiological apparatus used was essentially of the design used previously (DiBona & Civan, 1973) except that no feedback loop to correct for solution resistance was applied since all experiments employed identical mucosal and serosal baths. Voltage offset for clamping tissue potential at levels other than zero was applied through the positive input of one of the clamping amplifiers.

The final step in each experimental protocol was glutaraldehyde fixation of the tissue in the chamber. Appropriate volumes of 25% glutalardehyde were added simultaneously to serosal and mucosal baths to provide a 1% solution of fixative on either side of the tissue. The applied voltage clamp was maintained in the presence of the fixative for 15 to 30 min and in each case beyond the point where there was no further change in external circuit current. Subsequent processing of tissue was as before (DiBona & Civan, 1972) using a phosphate buffer at pH 7.4, post-fixation in OsO_4, alcohol dehydration and embedding in an Epon-Araldite resin.

Thick sections (1 μ) were stained with toluidine blue for light microscopy. Micrographs were obtained to cover comprehensively the strips of tissue obtained in blocks selected at random and coded for subsequent unbiased examination by each of the authors. Thin sections were stained with uranyl acetate and lead citrate and examined in either a Philips EM-200 or EM-301 electron microscope.

Results

Effects of Vasopressin on Short-Circuited Hemibladders

In a series of six experiments, paired quarter-bladders were short-circuited. Once short-circuit current had stabilized, vasopressin (100 mU/ml) was added to the serosal medium bathing one quarter-bladder while an equal volume of sodium Ringer's was added to the serosal medium of the control. When the stimulation in short-circuit current induced by the hormone had reached a peak value (15 to 20 min, after addition of the agent) both quarter-bladders were fixed by addition of glutaraldehyde. No detectable differences in cellular size were reported by six observers in any of the paired coded micrographs. The absence of detectable cellular swelling in these histological studies is consistent with findings from chemical analysis of vasopressin-treated hemibladders (Macknight, Leaf & Civan, 1971) where an increase in water content after vasopressin of 10% was reported. Such an increase would not be detected by the present technique and probably not by the morphometric methods used in the preceding study (Bobrycki et al., 1980). In the absence of an obvious effect on cell volume, we concluded that a cell-specific response could not be detected with this approach using short-circuited tissues.

Effects of Vasopressin on Tissue Clamped at −50 mV

From our previous study (Bobrycki et al., 1980) we have noted that in most, but not all, cases, clamping to −50 mV (serosa negative) resulted within 30–60 min in a marked swelling of granular cells. About one-third of these preparations proved resistant to swelling in experiments using this protocol and this resistance could be predicted on the basis of a stable external circuit current during the clamping interval. It was our impression as well that this resistance to

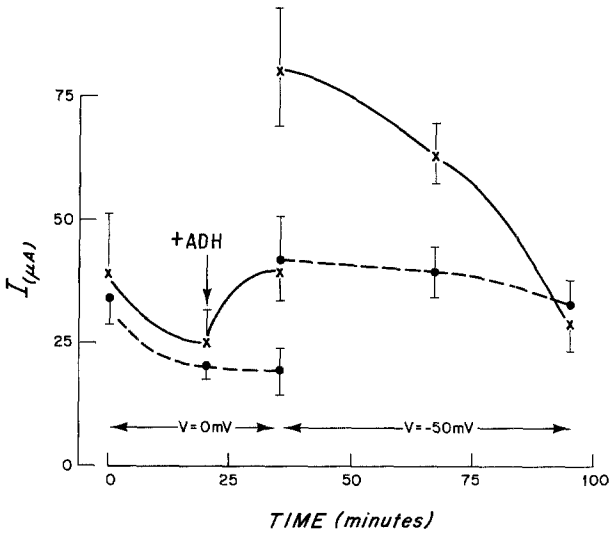


Fig. 1. Plot of external circuit current *vs.* time in six paired quarter-bladders with an experimental protocol as follows: after a 20-min equilibration period under short-circuited conditions, vasopressin (100 mU/ml) was added to the serosal medium of one tissue (solid line); an equivalent volume of Ringer's solution was added to the other. At the apparent maximum of the electrical response, both tissues were clamped to −50 mV (serosa negative), held for 60 min and finally fixed with glutarladehyde for microscopic examination

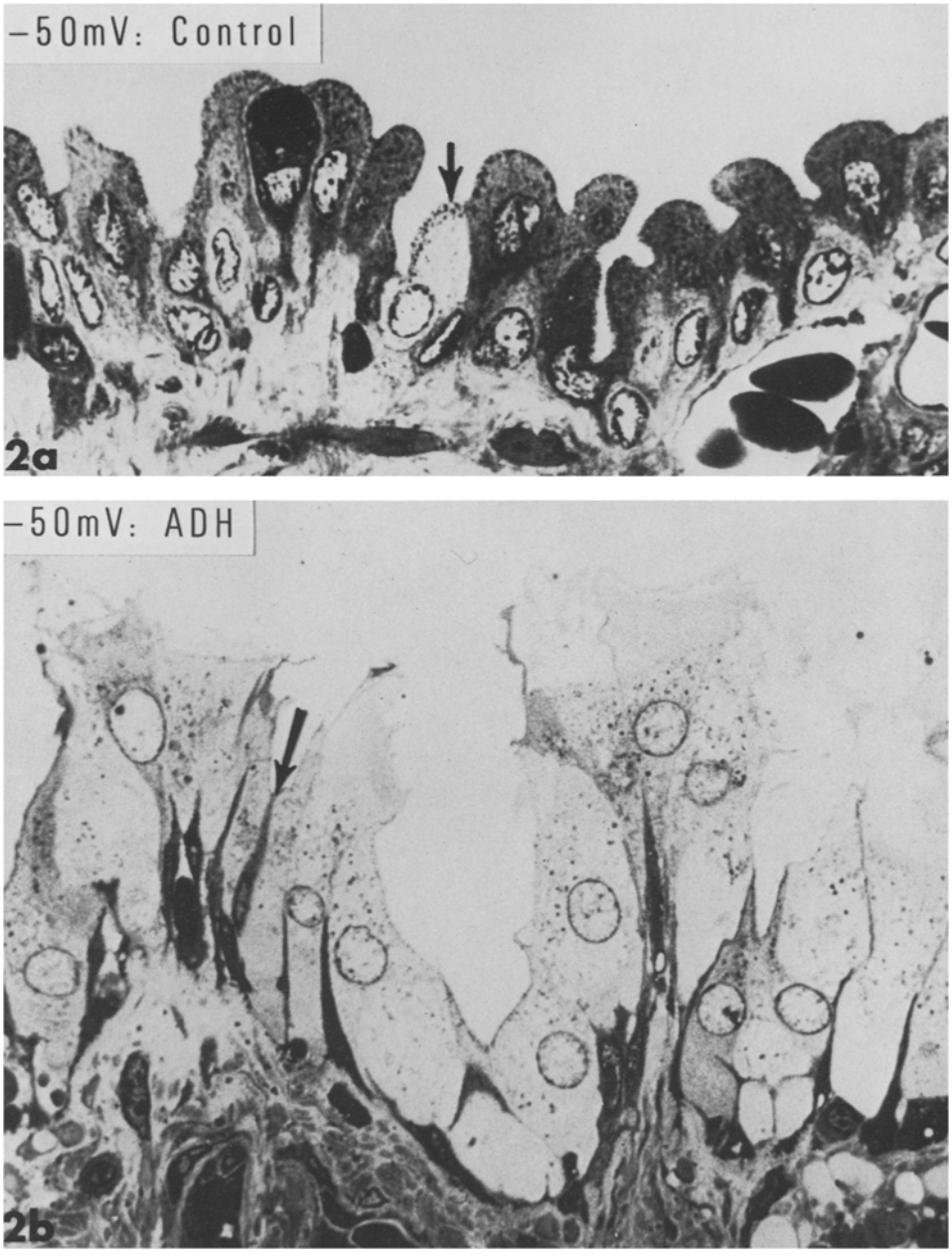


Fig. 2. Comparative light microscopic views of a pair of quarter-bladders from the protocol in Fig. 1. While only occasional granular cells were found swollen in the control preparation (Fig. 2*a*), gross swelling was evident in the presence of vasopressin (ADH, Fig. 2*b*) everywhere in this field. 700 ×

swelling varied from animal to animal; two hemibladders from a single animal routinely showed this resistance (or lack of it) to voltage-reversal. Based upon these observations, we were able to select a population of more resistant tissues by first conducting a trial run on one hemibladder from each animal. If this initial run showed stable external circuit current (less than a 20% fall over 60 min of depolarization to −50 mV) the second hemibladder was used in the protocol outlined in Fig. 1. After a 15–30 min baseline period for initial stabilization of current under short-circuited conditions, one quarter-bladder was stimulated with vasopressin (100 mU/ml to serosal bath). At the apparent peak of the response, both quarter-bladders were clamped to −50 mV and the current traces were monitored for an additional 60 min. As predicted from trial runs, quarter-bladders not exposed to hormone exhibited a nearly constant transmural current. Current traces for the vasopressin-treated tissues fell sharply with significant decreases ($p < 0.01$) of 22% and 64% by 30 and 60 min, respectively. After 60 min of voltage-clamping, the pairs were fixed by glutaraldehyde addition and processed for light and electron microscopy.

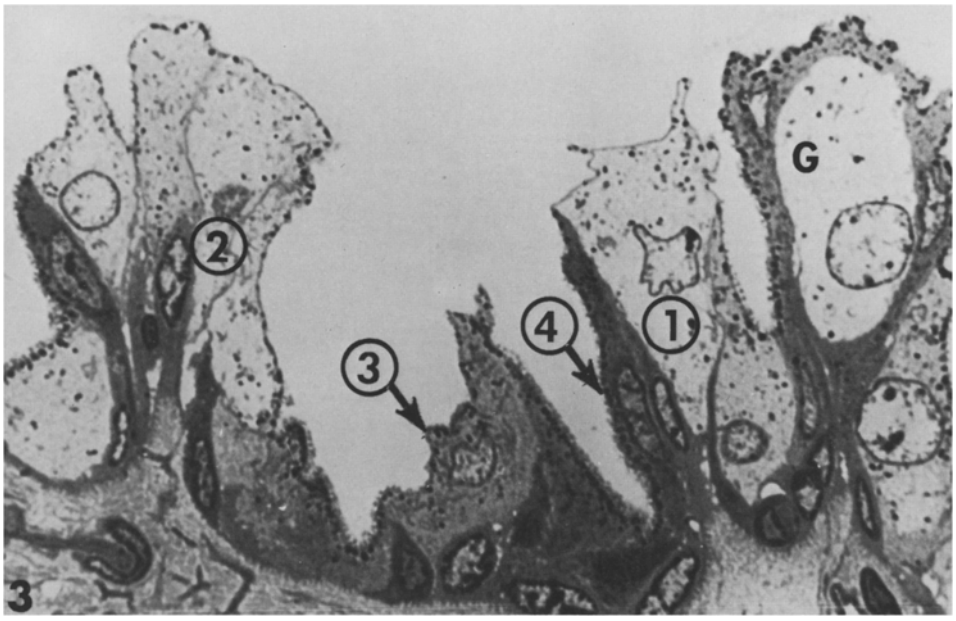


Fig. 3. Light micrograph of vasopressin-treated, voltage-reversed (−50 mV) preparation as in Fig. 2b, showing the wide variabilty in swelling among the granular cell population. Cell #1 has lost the characteristic microvillous mucosal margin and its nucleus seems to have lost some membrane integrity as the nuclear profile appears flaccid. Cells # 2, 3 and 4 show progressively diminished responses to the applied procedure. Goblet cells (*G*) were often dramatically swollen. 900×

Morphologic findings were very consistent and are illustrated in Fig. 2. As expected, untreated bladders from these animals appeared unaffected with only occasional swelling of a granular or a goblet cell in most of the tissue strips examined (Fig. 2*a*). Tissues treated with vasopressin were dramatically swollen, however (Fig. 2*b*). Expansion of granular cell volume was, in many cases, so severe that the epithelial lining was thrown into a pattern of folds projecting toward the mucosal bath. (This convolution of the surface is a logical consequence of increased cell dimensions along the epithelial plane in a space-filling array of cells.) The grossly swollen granular cells were the most apparent feature of these preparations but, as illustrated in Fig. 3, it is clear that some of the members of this cell population were far less affected. While there was no clear indication of swelling in identifiable mitochondria-rich cells, positive identification of these was extremely difficult given the level of disorganization in the swollen cells and the compressed appearance of those cells packed between swollen cells. For clarification of the distribution of swelling, electron microscopy was necessary.

Cellular Specificity and Fine Structure of the Vasopressin Effect

Figure 4 illustrates the marked difference between adjacent granular and mitochondria-rich cells which we found 60 min after voltage-reversal in the presence of vasopressin. It is not possible to conclude that there was no volume change in mitochondria-rich cells but positive evidence for swelling was lacking in the dozens of these cells examined. The pattern of changes in the granular cells was present in each of the preparations examined and always included a range of phenomena from apparently unaffected cells to those where virtually all internal organization seemed disrupted and the mucosal aspect of the plasma membrane had lost its characteristic microvillous appearance. The granular cell of Fig. 4 shows changes somewhere between these two extremes and contains a prominent bulge in the basal aspect of the cell as we had observed (Bobrycki et al., 1980) when swelling was induced by voltage-reversal in the absence of hormone. In Fig. 5*a*, where the granular cell is more dramatically affected, the adjacent mitochondria-rich cell appears normal, nonetheless. Here, as in the previous Figure, it is also clear that basal cells did not undergo a clear volume change in response to this treatment. When granular cells were expanded to this extent, localized distensions of the basal cytoplasm (as in Fig. 4) were generally not seen and the mucosal aspect of the plasma membrane had lost its microvillous appearance. Figure 5*b* is a higher power view of the mucosal aspect of strongly affected granular cells. The characteristic fibrous nature of the terminal web is evidently disrupted in these cells and, as shown

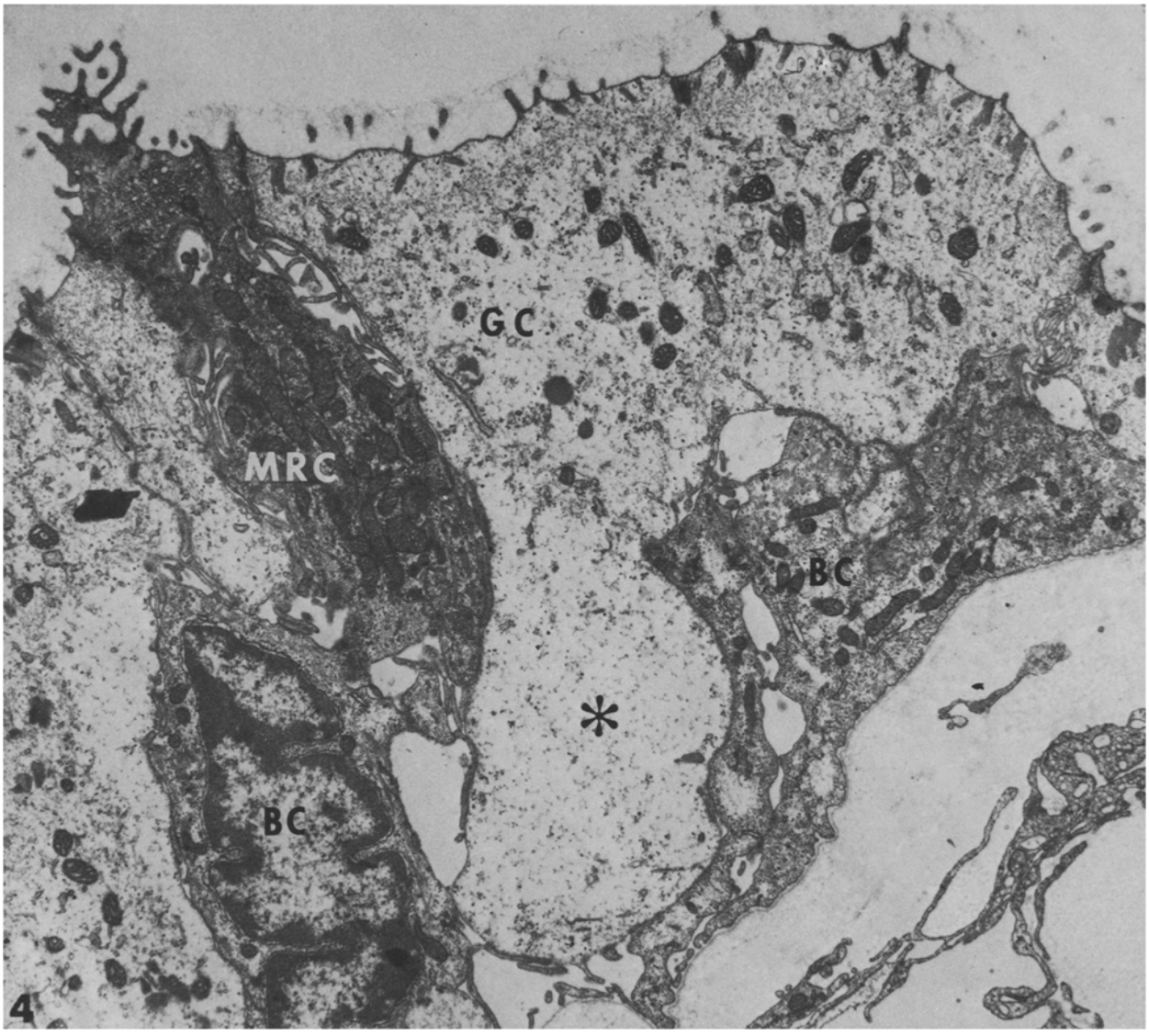
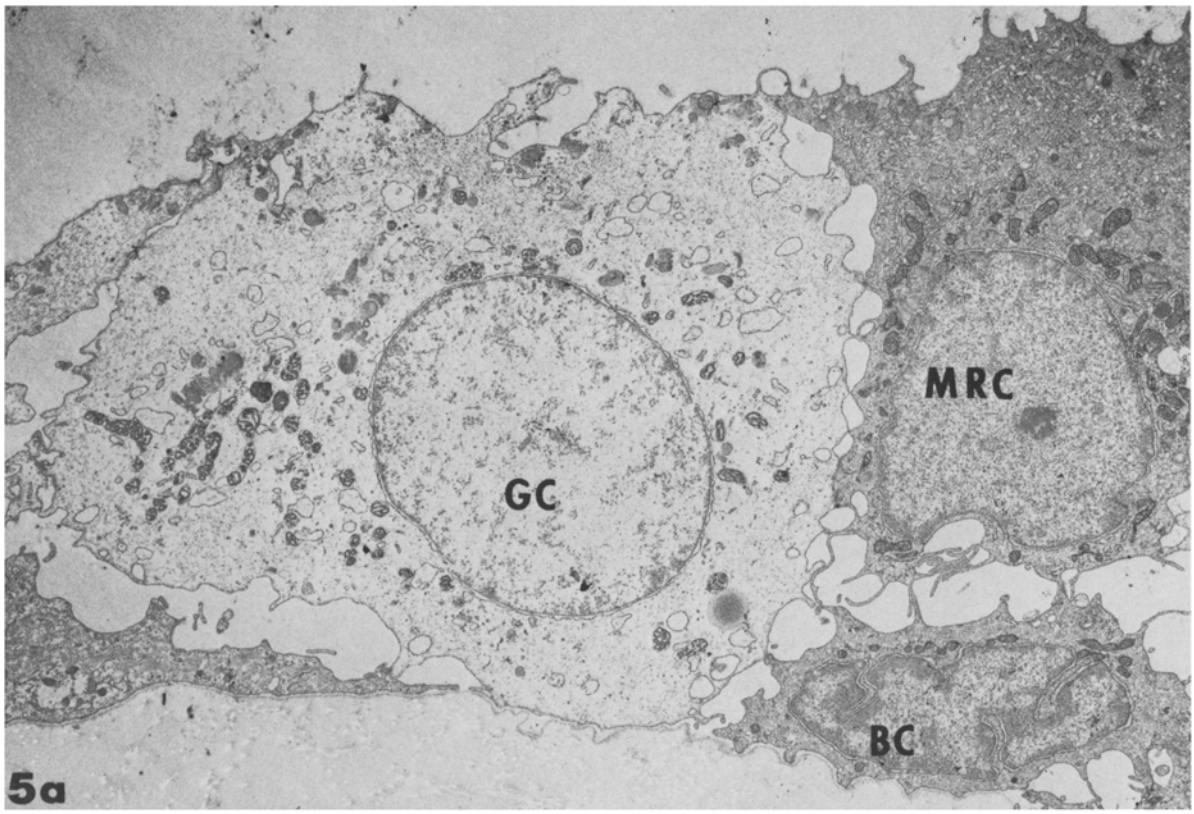


Fig. 4. Electron micrograph of vasopressin-treated. voltage-reversed (− 50 mV) bladder epithelium. Mitochondria-rich (*MRC*) and basal cells (*BC*) appear as they do in untreated (e.g. open circuited) preparations. The granular cell (*GC*) here is not swollen to the point of disruption in the apical cytoplasm but displays a focal distension (*) or bulge toward its basal margin. 9,500×

here, mitochondria were occasionally found close to the mucosal plasma membrane; in untreated tissues, these organelles are never found in the apical cytoplasm. Noteworthy is the observation of prominent distension in the outer membrane while the matrix of these mitochondria is condensed.

Vasopressin and Shunt Sensitivity to Depolarization

As is demonstrated in Fig. 5*a* and *b*, vasopressin-treated tissues subjected to voltage-reversal revealed blistered "tight" junctions to about the same extent as we had noted (Bobrycki et al., 1980) in the absence of hormone-stimulation. While no rigorous quantitative analysis was attempted, review of electron micrographs collected in this study suggests that 5–15% of the recorded junction profiles contained at least one discrete deformation. There was no apparent difference in blister-frequency between samples fixed in the presence of amiloride and those that were not. Junctional deformations seen here were indistinguishable from those we have previously seen in studies of paracellular shunt regulation by transmural osmotic gradients with mucosal hypertonicity (DiBona 1972; DiBona & Civan, 1973; Civan & DiBona, 1978). Current traces in voltage-reversal, vasopressin-



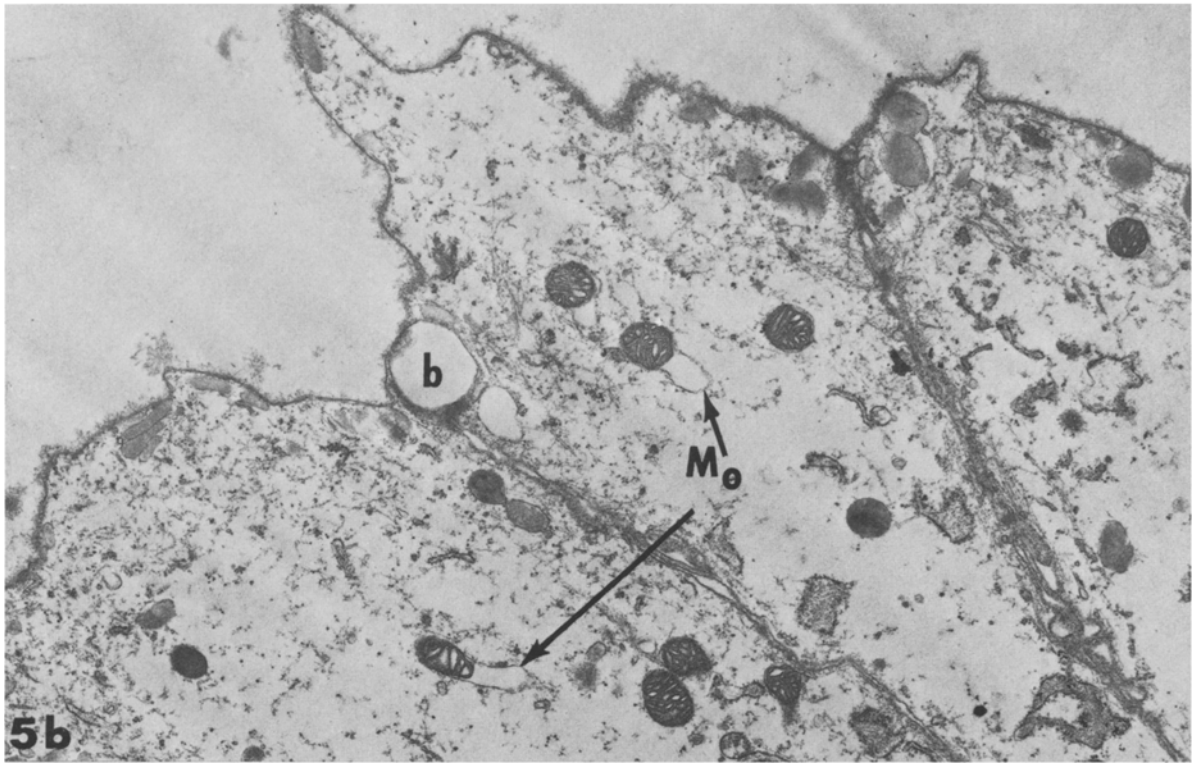


Fig. 5. Electron micrographs of severely affected granular cells after voltage-reversal in the presence of vasopressin. Note the loss of microvilli in the granular cell (*GC*) and the very irregular profile of the mucosal plasma membrane at the top of Fig. 5a; the mitochondria-rich (*MRC*) and basal cells (*BC*) appear unaffected, nonetheless. A prominent blister is present in the tight junction between the granular and mitochondria-rich cells. This is better seen between two granular cells in Fig. 5*b* (*b*) which also illustrates the distension of the mitochondrial outer membrane (M_o) that was routinely seen with this procedure. Fig. 5*a*: 5,300×; Fig. 5*b*: 12,500×

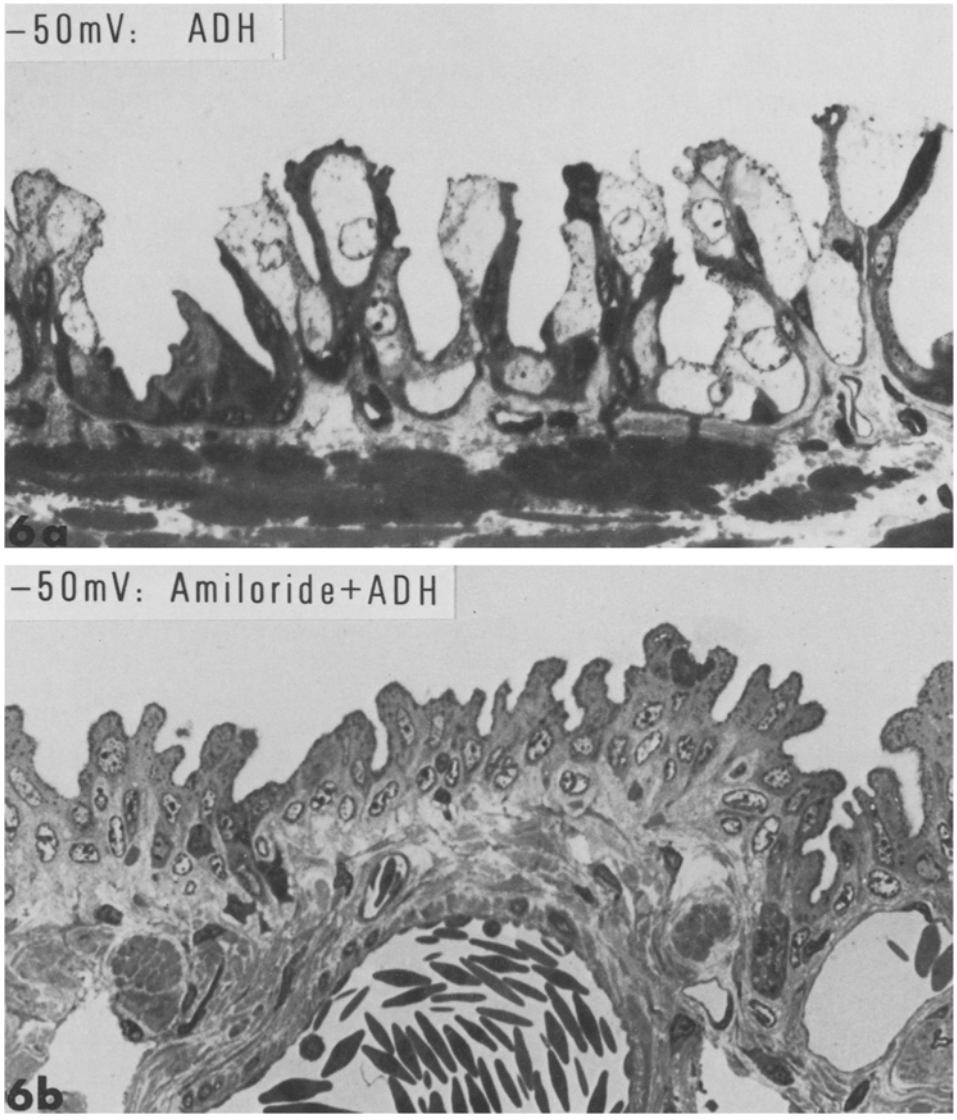


Fig. 6. Comparative light micrographs of voltage-reversed, vasopressin-treated quarter-bladder in the absence (Fig. 6*a*) or presence (Fig. 6*b*) of amiloride. These sections illustrate the uniform prevention of swelling that was observed when amiloride was applied under these conditions. 700×

stimulated tissue to which amiloride was added indicated a 15±3% increase in external circuit current over a 60-min interval. This, like the morphologic findings, is comparable to what has been seen without addition to hormone so that no major perturbation of shunt sensitivity to voltage-clamping can be ascribed to vasopressin itself.

Dependence of Vasopressin Action on Sodium Entry at the Mucosal Border

A major premise for this particular study was the conclusion of our previous work (Bobrycki et al., 1980) that granular cell swelling was contingent upon an inability of the active transport mechanism to move sodium out of the cell (basal-laterally) as fast as it was being driven in by the externally-enhanced electrochemical gradient across the mucosal plasma membrane. This conclusion was supported by the absence of swelling in the presence of the diuretic, amiloride. Following this reasoning, we employed amiloride in the present study as well. The protocol for six experiments involved addition of amiloride (10^{-4} M to mucosal bath) to one of a pair of short-circuited quarter-bladders at the approximate peak of their natriferic response to vasopressin. When current in the amiloride-treated tissue had fallen to within 1–2 μA of zero, each preparation was clamped to −50 mV (serosa negative) and the external circuit current followed for 60 min. Then tissues were fixed (as above) for subsequent microscopy. Over the 60-min clamping interval, external circuit current dropped by 71±6% in the quarter-bladder which had seen no diuretic,

and rose by $14 \pm 3\%$ in the amiloride-treated tissue. These results were in keeping with the first series described (Fig. 1) when external circuit current fell briskly in the presence of vasopressin. Similarly, in concert with the assumption of no hormone action on shunt-sensitivity, the external current at -50 mV rose to approximately the same extent as in our previous study (Bobrycki et al., 1980).

Microscopic examination of these samples revealed marked swelling of granular cells in each of six vasopressin-treated preparations where no amiloride was applied prior to voltage-reversal (Fig. 6*a*). The diuretic completely precluded this swelling; as demonstrated in Fig. 6*b*, none of these six quarter-bladders exhibited any obvious changes in granular cell volume.

Discussion

The major observation of this study is that granular cells of the toad urinary bladder become markedly swollen when vasopressin stimulation of the tissue is accompanied by a clamp of transmural potential to -50 mV (serosa negative). This swelling is found specifically within the granular cell population (mitochondria-rich cells are not noticably affected) and in the absence of any transmural osmotic gradient. Application of amiloride to abolish sodium entry to the cells from the mucosal bath completely precludes this volume change. These findings appear to support the proposition that vasopressin stimulates sodium transport in this tissue through an increase in the sodium conductance of the mucosal plasma membrane of the epithelial cells (Civan & Frazier, 1968; Macknight et al., 1971). Furthermore, the natriferic action of vasopressin appears to be mediated primarily, if not entirely, by the granular cells.

Basis for Granular Cell Swelling

Clear demonstration of the increase in granular cell volume with vasopressin depended on the use of tissues where voltage-clamping to -50 mV in the absence of hormone was without substantial morphologic effects. With tissues clamped at this potential, the rate of mucosal sodium influx is presumed to be very high but insufficient to exceed the capacity of the metabolically coupled extrusion step. As reasoned earlier (Bobrycki et al., 1980), cellular volume will only be increased after a "pump-saturating" level of transport has been achieved. The basis for this rests on the observation that cell volume in this epithelium does not seem to change in a linear way with the level of sodium transport. Rather, the capacity of the basal-lateral sodium pump is such that, over a wide range of transport activity, efflux to the serosa can comfortably accommodate sodium influx from the mucosa. Consequently, despite major differences in influx, we noted no cellular volume differences between open- and short-circuited tissues (Bobrycki et al., 1980). Recently, Canessa, Labarca, DiBona and Leaf (1978) reported that decreasing transepithelial potential difference from $+75$ to 0 mV increased active sodium transport by approximately 60%. Addition of vasopressin to short-circuited tissues, in the present study, increased measured current flow by an additional 60% and yet, no volume increase was observed. Since it seems that a two- to threefold increase in the rate of sodium entry to the cells is necessary before the active transport process becomes saturated, cellular swelling would not necessarily be expected. However, voltage-clamping vasopressin-treated tissue at -50 V initially increased current by an additional 100%. Although a small fraction of this increase reflects a flow through passive conductance elements in parallel with the active pathway, current flow through the active pathway has risen substantially and to an extent which now results in pump saturation, NaCl accumulation, osmotic uptake of water and gross cellular swelling.

The fact that amiloride completely prevented cell swelling, while blocking sodium entry at the mucosal margin, strongly supports the proposal that these effects are due specifically to an action of vasopressin on sodium transport. The hydroosmotic action of the hormone cannot be invoked for, in the absence of an osmotic gradient, increasing L_P cannot change volume.

The Electrical Response to Vasopressin and Voltage-Reversal

The decay in current following the voltage clamping of vasopressin-treated tissue at -50 mV (Fig. 1) was much greater than in control tissues. Since the clamping voltage was held constant, a decrease in current must reflect a decreased conductance across the swollen cells. As discussed in the previous paper (Bobrycki et al., 1980) the initial response in current may in part reflect the rate of accumulation of sodium chloride within the cells. As this process slows, current will decrease. In addition, increased cellular sodium concentration or decreased cellular potassium concentration may diminish apical membrane conductance (Erlij & Smith, 1973; Lewis, Eaton & Diamond, 1976; Robinson & Macknight, 1976; Cuthbert and Shum, 1977; Turnheim, Frizzell & Schultz, 1978; Helman,

Nagel & Fisher, 1979), and pump capacity itself may be decreased with time. This decrease in transport associated with cellular swelling induced by voltage-clamping and resulting from cellular accumulation of ions, should be contrasted with the increased current observed in frog skin (Ussing, 1965) and toad urinary bladder (Lipton, 1972) when cellular swelling is induced by a hypoosmotic serosal medium. Therefore, cellular swelling itself does not seem to provide a specific inhibition of transepithelial sodium transport.

Vasopressin and the Shunt Pathway

In swollen, voltage-reversed preparations exposed to vasopressin, bullous deformations (or blisters) of the "tight" junctions (*see* Fig. 5) were evident in 5–15% of the observed profiles, a frequency similar to that observed in the absence of the hormone (Bobrycki et al., 1980). In earlier work (Civan & DiBona, 1974), vasopressin stimulation prevented blister formation in the presence of a hyperosmotic mucosal medium. It was concluded that the increased water flow through the cells from the serosal to the mucosal medium had resulted in an increased osmolality within the lateral intercellular spaces, thereby diminishing the osmotic gradient across the junctions and minimizing junction deformation. In contrast, here, where the presumed driving force for blister formation is the externally applied voltage-clamp, any action of vasopressin on the mucosal membrane must be without effect on the voltage profile imposed across the junction. Consequently, the finding that blister formation is unaffected by the hormone is in keeping with its having no discrete action on the junction itself.

Vasopressin Action on Cell Conformation

The specific nature of the structural changes observed here may help to clarify the details of vasopressin action on the granular cells. When it was demonstrated that the granular cell was the specific cell involved in the response to the hormone (DiBona et al., 1969), it was noted that mitochondrial volume was not affected during the increased water flow, while the overall cell volume had increased markedly. Conversely, however, if cells were swollen as a consequence of dilution of the serosal medium, mitochondrial volume was increased dramatically. The response of the cell to these two experimental manipulations has been clarified in subsequent work (DiBona, 1979), where observations of the living bladder have established that swelling during ADH-induced osmotic water flow results in a bulging of the apical cytoplasm while the basal aspect of the cell may even be somewhat contracted causing prominent distensions of the intercellular spaces. In comparison, swelling of cells following dilution of the serosal fluid produced little or no distension of the apical cytoplasm while basal aspects of the cells were very much expanded. The changes in granular cell volume observed in the present experiments, suggest that, in addition to increasing permeability of the mucosal plasma membrane to sodium and water, vasopressin may directly affect cytoskeletal elements. This suggestion is based upon the observation that, in contrast to cells swollen by voltage-clamping alone, those swollen in the presence of vasopressin showed prominent apical bulging with loss of microvillous projections. Such bulging could reflect increased apical membrane compliance as a consequence of relaxation of the cytoskeletal elements which form a fibrous terminal web of actin-rich microfilaments (Rossier, Geering & Kraehenbuhl, 1979). In fact, vasopressin is known to cause relaxation of smooth muscle in this tissue (DiBona & Civan, 1972). Grantham (1970) measured an increase in apical membrane compliance in isolated cells of rabbit cortical collecting tubules after exposure to vasopressin. Similarly, observed alterations in microvillous conformation (Davis, Goodman, Martin, Matthews & Rasmussen, 1974; Spinelli, Grosso & de Sousa, 1975; Mills & Malick, 1978; DiBona, 1979; Dratwa, Le Furgey & Tisher, 1979) following vasopressin might be the result of changes in membrane compliance consequent upon an alteration in the tensile state of membrane-anchored actin.

In conclusion, the enhancement of granular cell swelling by vasopressin in voltage-reversed tissues and its prevention by amiloride strongly suggest that the effect of this hormone in the stimulation of transepithelial sodium transport is confined to the granular cells.

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