

MUCOSAL SURFACE MORPHOLOGY OF THE TOAD URINARY BLADDER

Scanning Electron Microscope Study of the Natriferic and Hydro-osmotic Response to Vasopressin

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ABSTRACT

The mucosal cell surface of the toad urinary bladder was examined by scanning electron microscopy, and changes in the structure of the surface of the granular cell were correlated with specific physiological responses to vasopressin. Survey views of the mucosal surface demonstrated that there was no consistent repeating anatomical relationship between the granular cell and the mitochondria-rich cell that would support the concept of cooperativeness in the response to vasopressin. During base-line states of Na^+ -transport and water flux, the microvilli on the mucosal surface of the granular cell are arranged in a ridge-like network with occasional individual projections. When water flux is increased by exposing the tissue to vasopressin, in the presence of an osmotic gradient across the tissue, the microvilli on the granular cell lose the ridge structure and appear, predominantly, as individual projections. Variability of this appearance points out the necessity of examining large areas and many samples before the significance of any morphological change can be assessed. Blocking the simultaneously occurring natriferic response of the toad urinary bladder with 10^{-2} M ouabain does not prevent these changes in the microvilli. When the hydro-osmotic response is blocked by eliminating the osmotic gradient, the granular cell shows no consistent change in mucosal surface morphology even when fixed at the height of the natriferic response. The mitochondria-rich and mucous cells did not show any change in morphology throughout these studies. We conclude that the changes in the mucosal surface morphology of the toad bladder seen after exposure to vasopressin are a result of the increased water flux that occurs when an osmotic gradient exists across the tissue, and are not related to the natriferic response or any specific alteration in the membrane properties.

KEY WORDS toad urinary bladder · scanning electron microscopy · vasopressin · granular cell The toad urinary bladder has long been used as a model system for studying the action of the poly-

peptide hormone vasopressin (2, 15). After exposure to vasopressin, the mucosal epithelial cells of the bladder undergo a marked increase in water permeability which results in an increase in bulk water movement in the presence of an osmotic gradient (the hydro-osmotic response). There is also a dramatic but transient stimulation of transepithelial Na^+ -transport (the natriferic response). The mucosal epithelium of the bladder is composed of four cell types (granular, mitochondria-rich, mucous, and basal) which, except for the basal cell, reach from the urinary space to the basal lamina (7). DiBona et al. (8) have proposed that the granular cell, the major cell type in the bladder, is the site of action of the hydro-osmotic response to vasopressin, as this was the only cell type to undergo a morphological change during vasopressin-stimulated water flux across the bladder. The cell responsible for the natriferic response could not be identified.

Recently, a series of investigations employing the scanning electron microscope (SEM) have described the mucosal surface morphology of the toad urinary bladder (4) and the appearance of this surface after the bladder has been exposed to vasopressin (5, 25). When toad bladders were stimulated by vasopressin in the presence of an osmotic gradient from the mucosal to serosal solutions, there was a marked change in the mucosal surface morphology of the granular cell. The most pronounced change was the transformation of a ridgelike network on the mucosal surface to the appearance of distinct microvillous processes. This alteration could be interpreted as being related to the increased water flux and concomitant increase in cell size induced by the hydro-osmotic response to vasopressin (25). However, because the change in the granular cell, observed with vasopressin in the presence of a gradient, was also reported to occur when bladders were exposed to vasopressin in the absence of a gradient (5), an alternative explanation is that the structural alterations are the result of a direct effect of vasopressin on the granular cell unrelated to the hydro-osmotic response. Also, the observation that the granular cell alone undergoes a morphological alteration when only the Na^+ -transport response can be expressed (absence of an osmotic gradient) provides indirect evidence that the granular cell is also responsible for the natriferic response.

The following report investigates this problem by employing the SEM to study the cell surface

morphology of the mucosal epithelium of toad urinary bladders fixed during different stages of the hydro-osmotic or natriferic response. We demonstrate that the granular cell is the only cell to undergo a morphological change after exposure to vasopressin, and that this change occurs when an osmotic gradient exists across the tissue.

MATERIALS AND METHODS

Urinary bladders were removed from doubly-pithed toads (National Reagents, Bridgeport, Conn.) and washed in isotonic Na^+ -Ringer's solution (Na^+ , 113; K^+ , 3.5; Ca^{++} , 0.9; Cl^- , 116; HCO_3^- , 2.4 mM; pH 7.5 to 8.0 and tonicity 220 mosmol/kg H_2O). Bladders were then stretched on a cork frame as a diaphragm and mounted in Ussing-type lucite double chambers. This procedure insured that the two areas of tissue within each bladder were stretched to the same degree.

Electrical measurement of Na^+ -transport was accomplished by a square wave voltage clamping routine (3) where transmural potential difference was alternately held at zero (short-circuited state) or 10 mV (serosal surface positive to mucosal surface). The current in the short-circuited state (the short-circuit current or SCC) is known to be an accurate measure of net transepithelial Na^+ -transport (26), and the difference in current between the two clamping voltages allows monitoring of transmural conductance.

In all cases, bladders were initially exposed to isotonic Na^+ -Ringer's solution on both sides, and the SCC was allowed to reach a steady state before any experimental protocols were begun. When it was necessary to establish an osmotic gradient, Na^+ -Ringer's on the mucosal side was diluted either 1:5 or 1:10 with distilled water. Ouabain (Sigma Chemical Co., St. Louis, Mo.) was added at 10^{-2} M. Vasopressin was added as Pitressin (Parke, Davis & Co., Detroit, Mich.) in a range of 40–200 mU/ml.

For water flux measurements, quarter bladders were tied, serosal side out, on glass tubes and filled with the appropriate Na^+ -Ringer's solution. The bags were then suspended in a beaker of isotonic Na^+ -Ringer's solution. Stirring and aeration was accomplished by bubbling room air into the beaker. The measurement of water flux was carried out by the technique of Bentley (2). Briefly, the glass rods with bladders attached were lifted out of the beakers, quickly blotted, and suspended from a hook on a Mettler balance (Mettler Instrument Corp., Princeton, N. J.). Weight loss, representing the flux of water out of the bag, was converted to microliters of water. Weighings were made every 15 min with a 1-h base-line period (Period 1). Then vasopressin was added to the serosal bath of each quarter bladder, and the water flux was measured over a 30-min period (Period 2). In the experiments with ouabain, the quarter bladders were next rinsed in three changes of fresh Na^+ -Ringer's solution for 1 h, and then a second base-line

period was determined (Period 3) with ouabain in the serosal bath of two of the quarter bladders. Vasopressin was then added to the serosal bath of all four quarter bladders, and a second 30-min response was monitored (Period 4). The values for the duplicate samples in each group were averaged.

At the end of the experimental period, concentrated glutaraldehyde was added simultaneously to both sides of each piece of bladder to a final concentration of 1%. Fixation was continued in place for 30 min, after which the tissues were removed and fixation continued overnight in a 0.1 M cacodylate buffer (pH 8.0) containing 1% glutaraldehyde. Tissues were washed in cacodylate buffer and postfixed in 1% OsO₄ for 1 h. For examination by SEM the bladders were treated as previously described (17). Tissues were dehydrated in increasing concentrations of ethanol and dried by the critical point method (1) using Freon 13.

RESULTS

Morphology

The morphology of the mucosal surface of the toad urinary bladder, fixed while exposed to Na⁺-Ringer's on both sides, is shown in Figs. 1 and 2. As previously described by Danon et al. (4), the large polygonal cells are the granular cells, the round cells with prominent microvilli are the mucous cells, and the small cells with densely packed microvilli are thought to be the mitochondria-rich cells. We found that the ratio of granular cells to mitochondria-rich cells was approximately 8:1, which is double the ratio reported by Danon et al. (4). This difference could be attributed to the previously reported variations in the number of mitochondria-rich cells in toads from different localities (21). Although each mitochondria-rich and mucous cell is associated with two to five granular cells, there are numerous granular cells that do not make contact with a mitochondria-rich or mucous cell (Fig. 1).

At higher magnification (Fig. 2), "fused" microvilli forming short rows of anastomosing ridges can be seen on each granular cell. This morphol-

ogy occurs in tissues bathed with isotonic Na⁺-Ringer's solution on both sides, or with an osmotic gradient of 1:5 or 1:10 from mucosa to serosa. This is in agreement with previous SEM studies of the toad bladder in which the tissues were examined after two different types of preparative techniques (5, 25). However, we found that this ridgelike appearance was never uniform throughout the epithelium. For example, on four adjacent granular cells seen in Fig. 3, the microvilli range from long ridges in one cell to predominantly single microvilli in another. This variation could be seen in adjacent fields as well as between individual cells.

The ridgelike appearance of the microvilli can be studied in the higher magnification view of Fig. 4. Here the diverse appearance of the ridges can be seen. In some cases they appear as continuous reflections of the cell surface. In other areas the ridges appear to be formed from adjacent individual microvilli making contact via the filamentous "fuzzy" coat. Although we were unable to determine conclusively if, indeed, the ridges represented a continuous fold of plasma membrane, examination of transmission electron micrographs show microvillar profiles that are much thicker than an individual microvillous process (inset, Fig. 4). These could represent a section through part of a ridge.

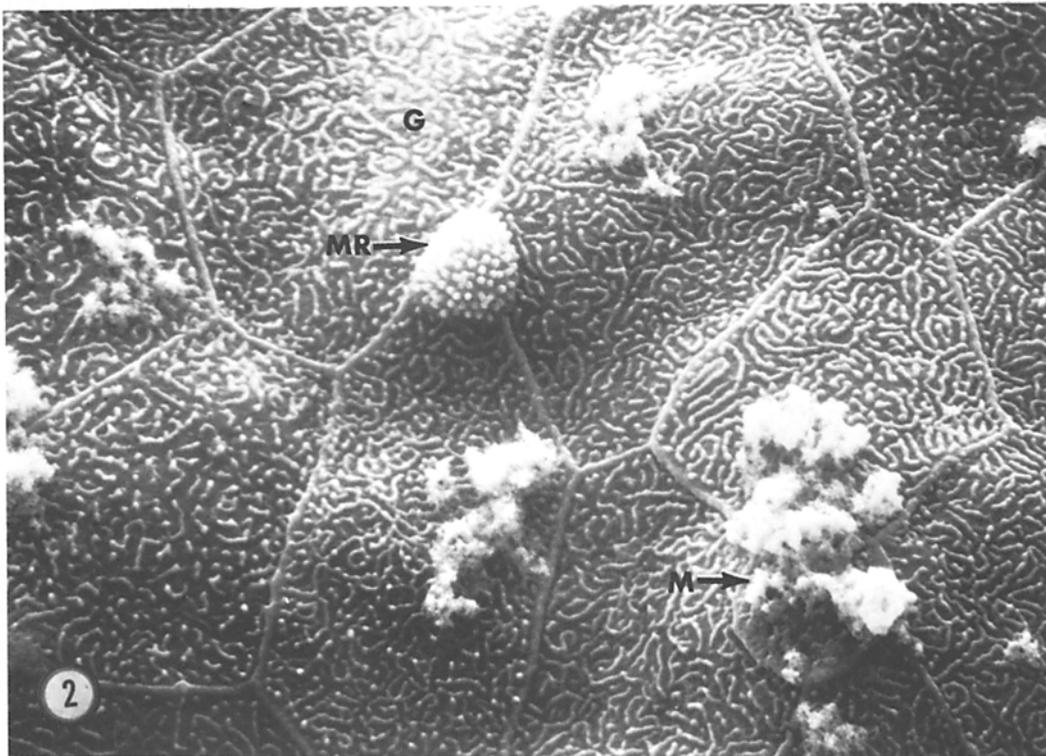
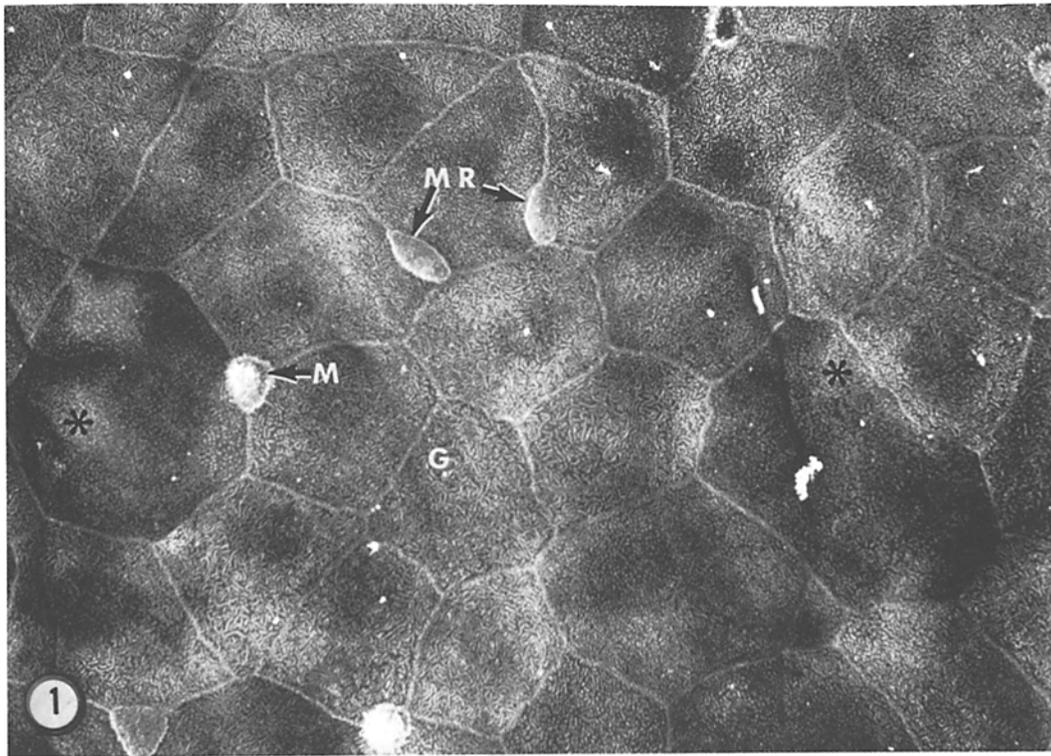
In some preparations the mucosal cells separated from each other or were lost, thus exposing the lateral cell membrane as well as the basal cell (Fig. 5). An important observation obtained from examination of these areas is that the lateral membrane of the granular cell, seen as a series of fingerlike projections in transmission electron micrographs, is arranged as a series of pleats or microfolds extending into the intercellular space.

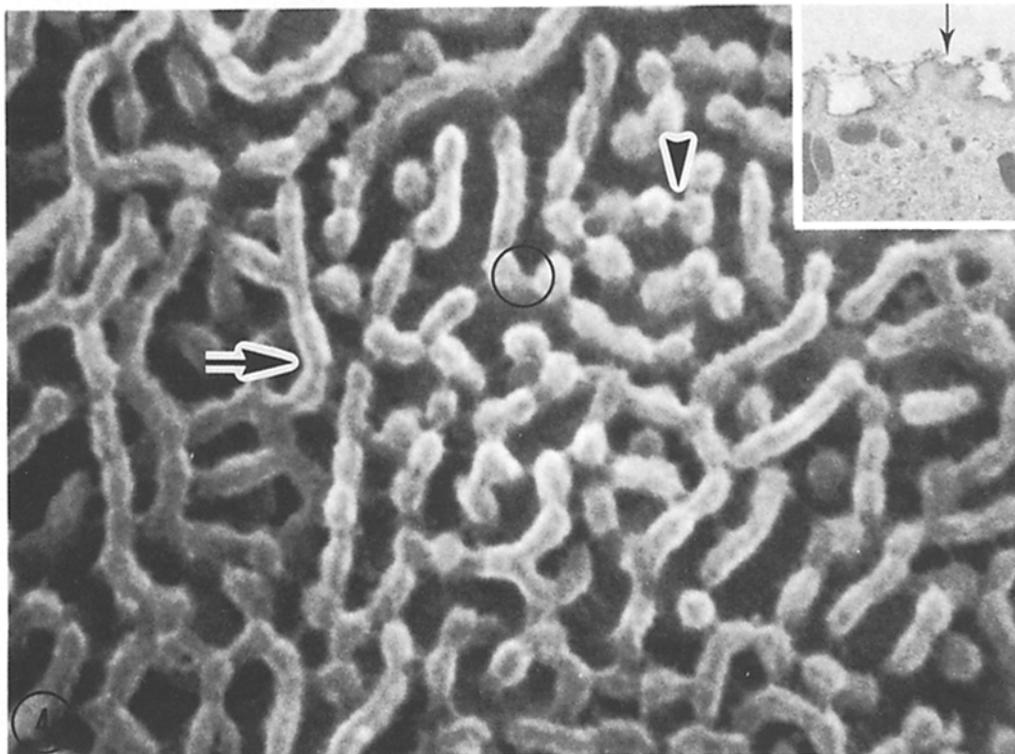
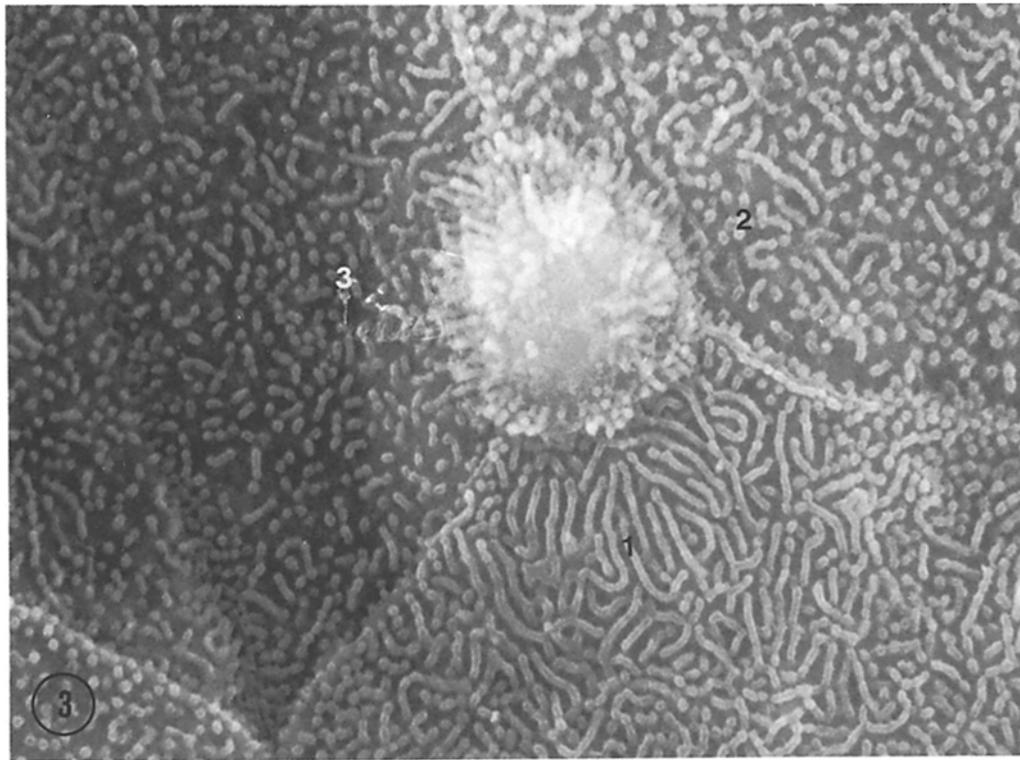
Effect of Vasopressin

When no osmotic gradient existed across the tissue, the mucosal surface of bladders, fixed at

FIGURE 1 A survey view of the mucosal surface of the toad urinary bladder showing the surface morphology and relationships of the different cell types. Notice that most of the granular cells (*G*) have a ridgelike appearance, although on some cells the microvilli are seen as individual projections (stars). There are nine granular cells that do not make contact with either a mucous (*M*) or mitochondria-rich cell (*MR*). $\times 1,240$.

FIGURE 2 The mucosal surface of the toad urinary bladder. The surface of the granular cells (*G*) is characterized by ridges of various length mixed with a few individual microvilli. The junction between granular cells is distinguished by an elevated ridge formed from the close apposition of two rows of microvilli. *MR*, mitochondria-rich cell; *M*, mucous cell. $\times 2,610$.





the height of the natriferic response, had a morphology similar to bladders which were never exposed to vasopressin (Fig. 6). This was also the case if the bladders were fixed 30 min after the addition of hormone when the SCC was declining back to base-line values. Bladders examined after exposure to hormone in the presence of an osmotic gradient showed changes that were limited to the granular cell and these changes were most evident after a 30-min exposure period (Fig. 7). In most cases, the granular cells showed signs of extensive swelling (Fig. 7). The amount of this swelling, visible from the luminal side, appeared to be related, to some extent, to the degree of stretching of the bladders before mounting. In highly stretched preparations the granular cells did not bulge outward to any appreciable degree (Fig. 12). The frequency of granular cells with a ridgelike appearance on the mucosal surface was greatly reduced in bladders exposed to hormone in the presence of an osmotic gradient (Fig. 8). This was especially evident in tissues exposed to a 1:10 osmotic gradient from mucosa to serosa (Fig. 9). Again, however, no single bladder which had been exposed to vasopressin in the presence of an osmotic gradient was uniform in appearance throughout. Ridges of microvilli could be found in some cells even when the gradient was 1:10 or the tissues were exposed to supramaximal doses of vasopressin (Fig. 10).

A third series of experiments was conducted to further determine if the change in appearance of the microvilli was related to the hydro-osmotic response of the tissue. In the first group, the pieces of urinary bladder in both sides of the chamber were exposed to a 1:5 osmotic gradient, and then SCC was reduced to zero by the addition of 10^{-2} M ouabain. One bladder of each pair was then exposed to vasopressin for 30 min, and the tissues were fixed. Fig. 11 shows the mucosal surface of bladders exposed to 10^{-2} M ouabain in

the presence of a 1:5 gradient. The morphology is similar to that of other bladders where the SCC was either at base-line values (Fig. 2) or at the peak of the natriferic response (Fig. 6). Fig. 12 is the paired hemibladder of Fig. 11. In addition to being exposed to 10^{-2} M ouabain until SCC reached zero, the tissue shown in Fig. 12 was also subsequently incubated with vasopressin for 30 min. The microvilli are seen as individual extensions of the cell surface with almost complete loss of the ridgelike appearance.

Because it has been reported that prolonged exposure of the toad urinary bladder to high concentrations of ouabain (10^{-4} M) inhibited the hydro-osmotic response to vasopressin (9), an extensive series of water flux measurements were made in sacs treated with 10^{-2} M ouabain. As shown in Table I, after a 60-min exposure to 10^{-2} M ouabain, the toad bladder still elicited a very strong hydro-osmotic response to vasopressin. This exposure time was much longer than was needed to completely inhibit the natriferic response to vasopressin as measured by the SCC in chamber experiments. When the period 4 value was expressed as a fraction of the period 2 value (no ouabain), the ouabain-treated bladders showed a slightly decreased response (0.84–0.71). However, these values were not significantly different when analyzed by the *t* test for paired samples.

A similar series of experiments was also done in which all the Na^+ in the solution bathing the mucosal side was replaced with choline. However, even after extensive washings to remove Na^+ , there was always a slight increase in the SCC after the bladder was exposed to vasopressin, and thus a purely hydro-osmotic response could not be elicited.

DISCUSSION

The ridgelike appearance of the apical surface of

FIGURE 3 An area of the mucosal surface of the granular cell showing a mucous cell and portions of four granular cells. The configuration on the granular cells varies from numerous ridges (cell 1) to a mixture of ridges and a various number of individual microvilli (cells 2 and 3). $\times 7,480$.

FIGURE 4 A high magnification view of the mucosal surface of the granular cell. Some of the microvilli appear as continuous ridges along the apical membrane (arrow) and others are individual projections from the apical surface. In some cases the ridges are actually formed from individual microvilli which make contact via a filamentous coating or "fuzz" (arrowhead) $\times 25,120$. *Inset*, transmission electron micrograph showing a microvillar profile which is twice as thick (arrow) as an individual microvillar projection. This view may have been the result of a section through a microvillous area as enclosed in the circle in the main figure.

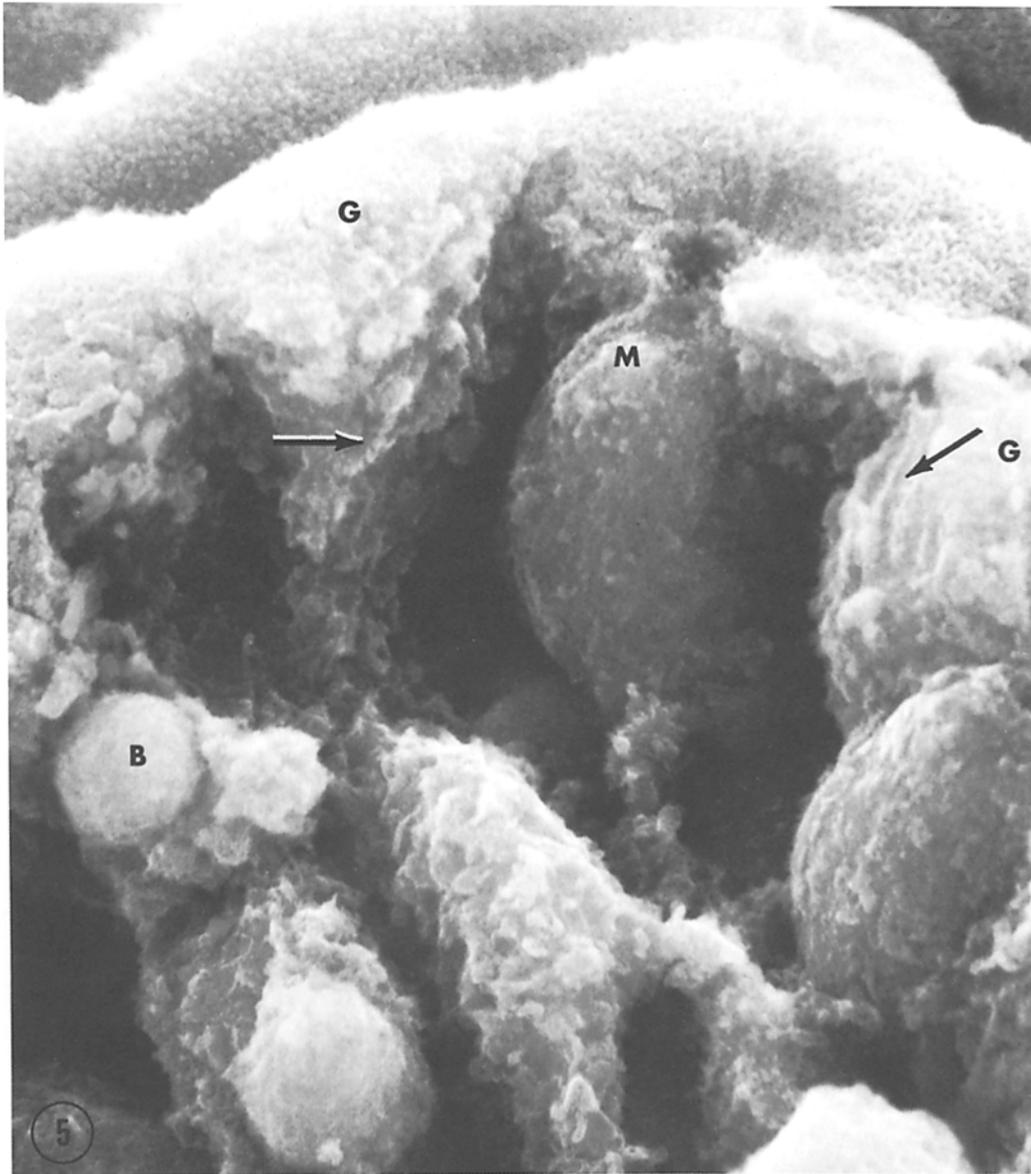
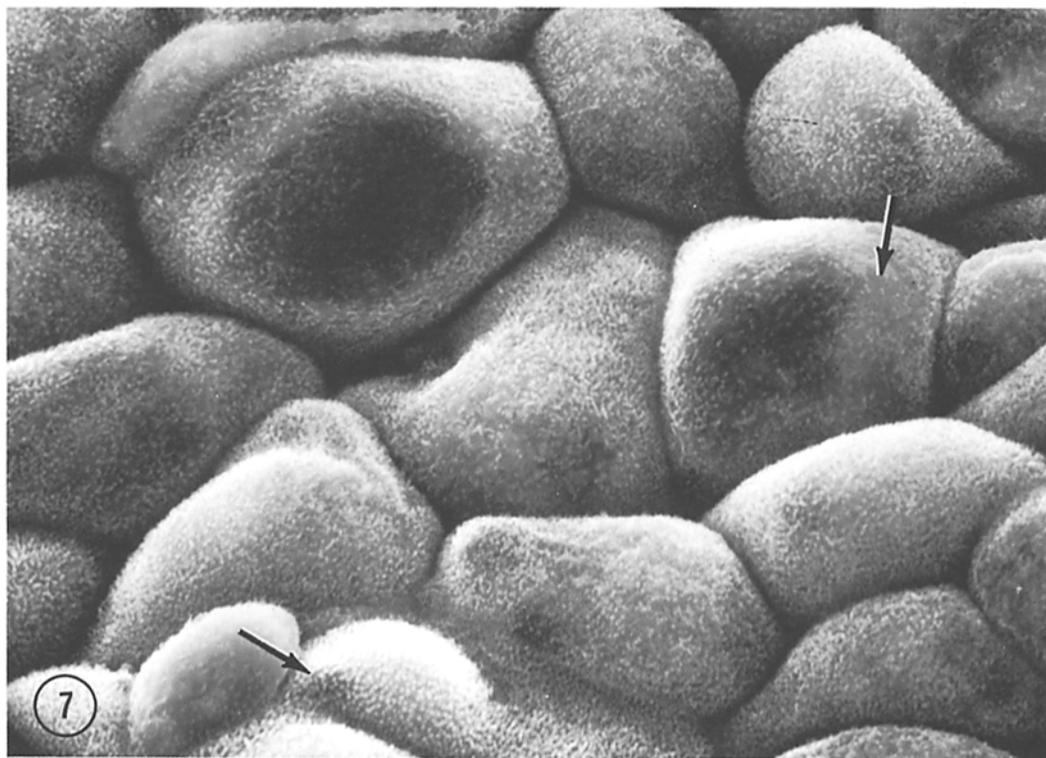
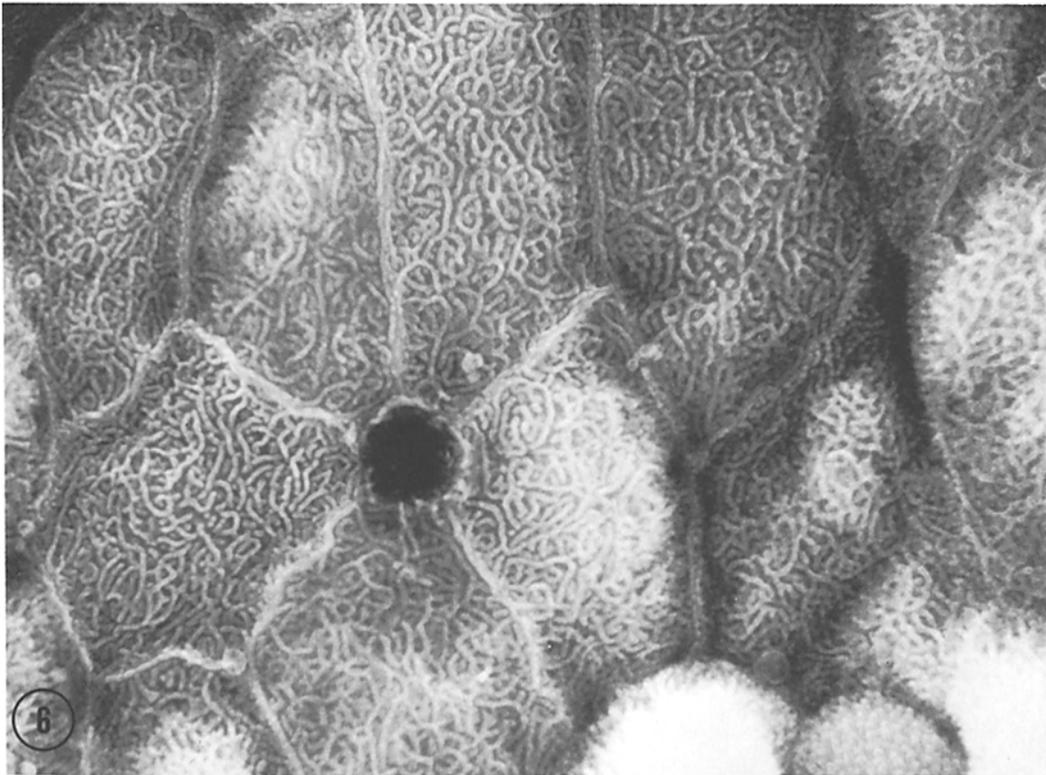


FIGURE 5 A lateral view of the mucosal epithelium of the toad urinary bladder. This view was made possible by the loss of the neighboring cells during processing. Notice that the lateral membrane of the granular cell (*G*) is arranged in a series of folds which form extensive ridges all along the cell from the apex to the base (arrows). In contrast, the mucous cell (*M*) has small individual extensions into the intercellular spaces. *B*, basal cell. $\times 4,500$.

FIGURE 6 The mucosal surface of the toad urinary bladder fixed at the point where the SCC had begun to plateau after exposure to 40 mU/ml of vasopressin (14 min after exposure to hormone for this bladder). No osmotic gradient was present. The surface of the granular cells has a morphology similar to Fig. 2 which was not exposed to vasopressin. The opening of a mucous cell is in the center of the field. $\times 4,960$.

FIGURE 7 The mucosal surface of the toad urinary bladder fixed 30 min after exposure to 40 mU/ml vasopressin and with a 1:5 osmotic gradient present. The granular cells appear swollen and the ridges are greatly reduced (arrows). $\times 3,000$.



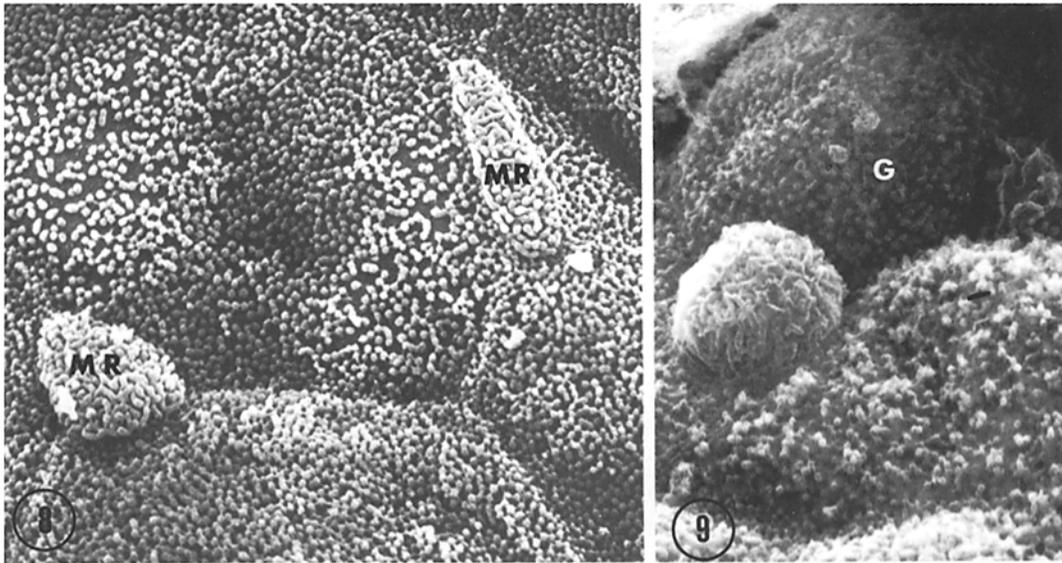


FIGURE 8 A higher magnification view of the mucosal surface of the toad urinary bladder fixed under the same conditions as in Fig. 7. The microvilli are seen predominantly as single projections of the cell surface. Notice that there are still many filamentous connections between individual microvilli. *MR*, mitochondria-rich cell. $\times 3,600$.

FIGURE 9 Mucosal surface of the toad urinary bladder fixed after exposure to 40 mU/ml of vasopressin. A gradient of 1:10 mucosa to serosa existed across the tissue. Microvilli on the granular cells (*G*) appear predominantly as single projections of the cell surface. $\times 3,400$.

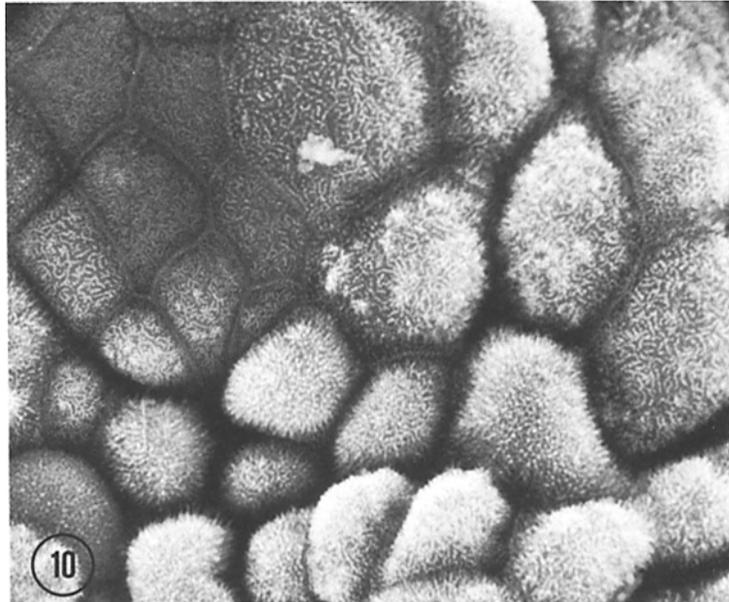


FIGURE 10 Mucosal surface of the toad urinary bladder fixed after a 30-min exposure to 200 mU/ml of vasopressin. A gradient of 1:5 mucosa to serosa existed across the tissue. In some cells the microvilli have a ridgelike appearance (even when swollen) while microvilli of other cells appear as individual extensions of the cell surface. $\times 1,580$.

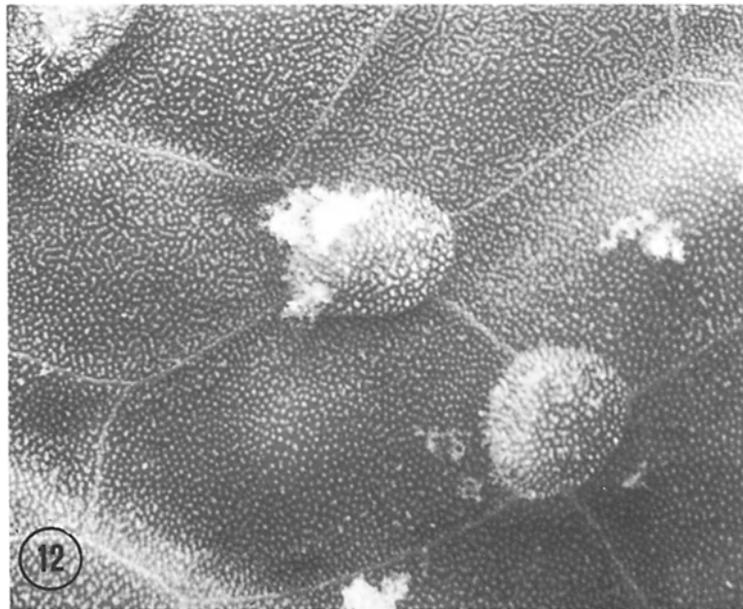
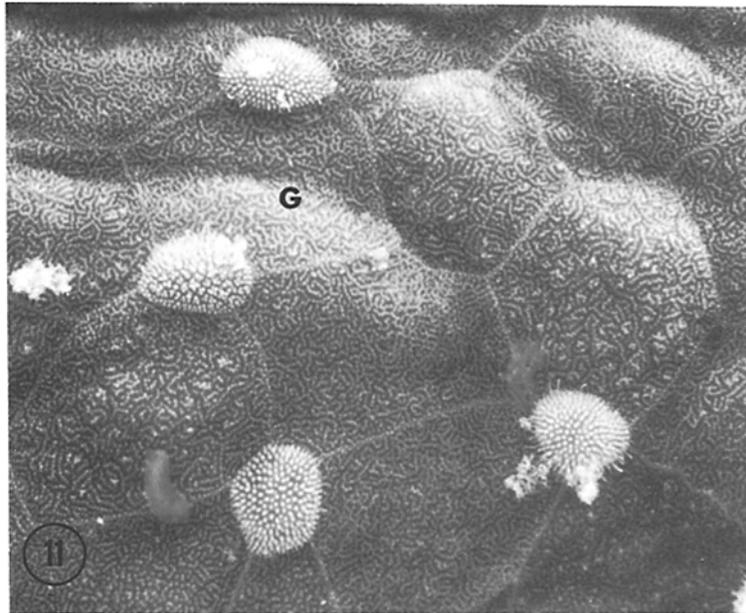


FIGURE 11 Mucosal surface of the toad urinary bladder fixed after an exposure to 10^{-2} M ouabain. The SCC had been at zero for over an hour. A gradient of 1:5 mucosa to serosa existed across the tissue. The surface of the granular cells (G) is similar to those in Figs. 2 and 6. $\times 1,455$.

FIGURE 12 Mucosal surface of the toad urinary bladder treated as in Fig. 11 except that the tissue had been exposed to 40 mU/ml of vasopressin for 30 min. Notice that the granular cells show almost complete loss of ridges. There is also no apparent luminal swelling of the granular cells. $\times 2,016$.

the granular cell of the toad urinary bladder was first described by Davis et al. (5). Microridges have been reported on the apical surface of many epithelial cells (24). Wassersug and Johnson (27)

proposed that the ridges may be a reserve of surface membrane to be utilized during stretching. This could be the function of the ridges on the toad urinary bladder, as Gfeller and Walser (10)

TABLE I
Water Flux Across the Toad Urinary Bladder in the Presence of a 1:5 Osmotic Gradient from Mucosa to Serosa

	Period 1	Period 2	Period 3	Period 4
			(μ H ₂ O)	
Control	22.6 \pm 3.9	617.1 \pm 75.9	17.8 \pm 8.8	517.9 \pm 55.9
Ouabain	29.9 \pm 3.9	924.3 \pm 143.8	36.6 \pm 6.0	656.9 \pm 109.8

Period 1 is a 1-h base-line period followed by a 30-min exposure to vasopressin (Period 2). After a 1-h wash, one pair of quarter bladders was exposed to 10^{-2} M ouabain and a second 1-h base line for each quarter bladder was monitored (Period 3). Then a second 30-min response to vasopressin was determined (Period 4). $n =$ six animals \pm SEM.

demonstrated that the microvillar profiles on the granular cell, seen by transmission electron microscopy, are gradually reduced in height as the bladder is stretched. However, this cannot be the function of the ridges on every cell where they occur, as extreme stretching of the esophageal mucosa of salmon (24) and of the guinea pig urinary bladder (28) did not change the appearance of the apical cell surface.

Although the ridges on the granular cell of the toad urinary bladder appear, in many cases, to be continuous reflections of the apical cell surface, examination at high magnification suggests that at least some of these ridges are individual microvilli joined together by the filamentous "fuzzy" coat. We were unable to determine conclusively if the joining of the microvilli was the actual configuration in the living tissue or was caused by preparation techniques. However, the presence of neighboring cells with different configurations on the apical surface (Fig. 3) provides evidence that the presence or absence of ridges may reflect differences in the physiological state of the individual cells.

The appearance of the lateral membrane of the granular cell as a series of microfolds has also been described for the lateral membrane of gallbladder epithelial cells (19). This observation is important because when the width of the lateral intercellular spaces is relatively small, the presence of these folds would greatly increase the effective channel length. In models which attempt to refine the theory of solute-linked water movement across epithelia (6, 14, 22), channel length has various degrees of influence on the tonicity of the emerging absorbate. In addition, the presence of folds, rather than microvillous projections, would greatly increase the lateral membrane surface area over which equilibration (related to the osmotic water permeability) with the intercellular

space could take place. The actual "effective" channel length and lateral membrane surface area are important values to determine in transporting epithelia if one is to adequately test the models of solute-linked water movement which treat the lateral intercellular spaces as cylinders (6, 14) or rectangles (22).

Mitochondria-rich cells were always found in association with three to five granular cells. Goodman et al. (11) have suggested that a repeating anatomical relationship of this kind may be important for intercellular chemical transfer of cyclic AMP. Handler et al. (12) reported that vasopressin increases the content of cyclic AMP in the mucosal epithelium of the bladder, and there is strong evidence that the action of vasopressin is mediated by this cyclic nucleotide (20). In toad bladder epithelial cells separated by Ficoll density centrifugation, the increase in cyclic AMP could only be detected in the mitochondria-rich cell fraction (23). Thus, if the receptor for vasopressin is the mitochondria-rich cell and the hydro-osmotic response occurs in the granular cell, cyclic AMP must diffuse very rapidly from the mitochondria-rich to the granular cells (11). A repeating anatomical arrangement of one mitochondria-rich cell in contact with several granular cells would satisfy the structural requirement for such a mechanism.

We believe that this proposed mechanism is unlikely on the basis of the anatomical evidence presented here, as well as studies by others on isolated, separated toad bladder epithelial cells (13). First of all, not every granular cell makes contact with a mitochondria-rich cell. In fact there can be groups of 7-10 cells which do not contact a mitochondria-rich or mucous cell. In addition, mucous cells have a similar anatomical association with the granular cell, and there is no evidence that they play any role in the response to

vasopressin. Finally, Handler and Preston (13) have raised a question as to the viability of toad urinary bladder cells separated by Ficoll density centrifugation, and the reliability of results obtained in studies of the isolated cell fractions.

Our observations on the changes in the granular cell mucosal surface agree with Spinelli et al. (25), and are at variance with those of Davis et al. (5). The change from the ridgelike to a microvillous surface was consistently observed in toad bladders exposed to vasopressin in the presence of an osmotic gradient, a condition which allows the hydro-osmotic response to be elicited. When no gradient existed, and thus only the natriferic response could be elicited, the ridgelike arrangement on the mucosal surface of the granular cell was the predominant condition. This was the case whether Na^+ -transport was zero, at base-line values, or maximally stimulated by vasopressin. It should be emphasized, however, that in almost every bladder examined the two extreme configurations, as well as intermediate conditions, could be observed.

Further evidence that the changes seen in the granular cell were a result of the increased water flux caused by the hydro-osmotic response to vasopressin comes from the experiments with ouabain. After 10^{-2} M ouabain, added to the serosal medium, reduced the SCC to zero, vasopressin failed to elicit a natriferic response, while in the presence of an osmotic gradient, a hydro-osmotic response persisted. Under these conditions the change in the appearance of the mucosal surface of the granular cell was identical to those observed in tissues in which conditions favoring both the natriferic and hydro-osmotic response existed. Thus we conclude, as did Spinelli et al. (25), that the changes observed are related to the increased water flux across toad bladders exposed to vasopressin in the presence of an osmotic gradient. We cannot explain the observation of Davis et al. (5) that the changes in the granular cell occurred whether or not a gradient existed. However, Macknight et al. (16) showed that the water content of the mucosal epithelial cells of the toad bladder increased by about 10% after exposure to vasopressin when both sides of the bladder were bathed with isotonic Na^+ -Ringer's. Thus, one might argue that this increase in volume may be enough to alter the mucosal surface morphology, at least in some cells, to appear similar to bladders in which a large net flux of water is occurring. In any case, the results of our study point out the

necessity of studying large areas of each bladder, as well as many bladders, before the significance of any morphological change can be assessed.

This study provides further support for the proposal by DiBona et al. (8) that the granular cell is the site of the hydro-osmotic response to vasopressin. The cell type responsible for the natriferic response is still not known. However, because the granular cell has a large number of Na^+ -pump sites (18), it may also be responsible for the rapid but transient increase in Na^+ -transport observed after exposure of the toad bladder to vasopressin.

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