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The Hydrosmotic Effect of Vasopressin: A Scanning Electron-Microscope Study

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Summary. Scanning electron-microscopy (SEM) was used to investigate the hydrosmotic effect of vasopressin on the apical surface of urinary bladders of toads *Bufo marinus*. Bladders were mounted on glass chambers and water fluxes were monitored with an optical method. Tissues were fixed in 2% glutaraldehyde and processed for SEM. Three types of cells were seen on the surface of control bladders: large polygonal (granular) cells, with blunt microvilli; smaller (mitochondria-rich) cells, with longer microvilli; goblet cells. Neither exposure of the bladders to a large osmotic gradient nor exposure to vasopressin in the absence of a gradient altered appreciably the epithelial surface. In contrast, the combination of vasopressin and an osmotic gradient resulted in a conspicuous diminution of the blunt microvilli. However, the small cells with longer microvilli remained unchanged. Identical results were seen with cAMP or theophylline in the presence of an osmotic gradient. These findings suggest that the hydrosmotic effect of vasopressin is mainly exerted on the granular cells of toad bladder and confirm observations made by others with the electron-microscope.

It is well known that vasopressin increases the transepithelial transport of several chemical species, namely sodium, urea and water. Its mode of action has been particularly well-studied in toad bladder and efforts have been made to define pathways and mechanisms of transport and to characterize the impact of hormones on such phenomena.

However, despite a considerable amount of work in this field in recent years, uncertainties persist as to the specific role of the different cell types of an epithelium in respect to a given transport process. Concerning water transport, there has been some controversy about the major route of the hydrosmotic flow across a "tight" epithelium such as toad bladder. Two main possibilities have been considered: (1) an intercellular pathway, through the tight junctions; and (2) a cellular pathway through the plasma membranes. The work of Civan and associates has produced convincing evidence in favor of the cellular pathway [4, 10]. The presence of three

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different types of cells in contact with the lumen of the bladder raises another question; that is, do all cells participate in the hydrosmotic flow across the epithelium, or is this a particular function of a given cell type? The results obtained by Di Bona, Civan and Leaf [10] with the electron-microscope (EM) strongly suggest that the granular cells of the bladder are the major route for bulk flow of water.

The advent of the scanning electron-microscope (SEM) makes available a new and versatile tool for the study of cell and tissue biology [21]. This technique seems to be particularly suitable for investigating the surface topography of epithelia and has been recently applied to turtle bladder [18] and toad bladder [5, 6, 11].

In this paper we report the results of a morpho-functional study with SEM using the bladders of the toad *Bufo marinus*. The bladders were exposed to different combinations of osmotic gradient and to stimulation of water flow with vasopressin, cyclic AMP and theophylline. A preliminary report of these results has appeared elsewhere [14].

Materials and Methods

Toads (*Bufo marinus*) were obtained from Mogul-Ed Co., Oshkosh, Wisconsin, and kept in a terrarium provided with a water basin. The temperature of the room averaged 22 ± 2 °C. A small area of the terrarium was kept at a higher temperature (27–28 °C) by means of an infrared lamp. The animals were fed once a week, either with mealworms or newborn rats.

After doubly pithing the toads, the urinary bladders were excised, quartered and the quarter-bladders mounted on glass chambers. The water flow measurements were performed by means of an automatic, optical method [7, 19]. With this technique, the movement of the meniscus inside a horizontal pipette is followed continuously by means of a carrier having attached to it a light source, a photosensitive element and a pen. The movement of the carrier, triggered by the optical system, is parallel to that of the meniscus and is directly recorded on a strip-chart recorder. From the cumulative water flow curve thus obtained, the transepithelial flux, expressed in μliters $min^{-1} \cdot cm^{-2}$, is readily calculated [19].

The bladders were exposed to Ringer's solutions of standard composition [1]. To establish an osmotic gradient across the membrane, the mucosal bathing solution was diluted five- to 10-fold with distilled water.

After completion of the functional studies, the quarter-bladders were quickly removed from the chambers and fixed with a solution of 2% glutaraldehyde containing a phosphate buffer (pH = 7.4). The tissues were then mounted on a sheet of dental wax, with the mucosal surface facing upwards. They were kept in this position with staples placed near the edges and immersed in a glutaraldehyde solution of the same composition as described above until processed for SEM observation.

Preparation of the bladders for SEM observation consisted of dehydration with graded acetones followed by air drying in a desiccator. The samples were then placed on aluminum holders and coated, under vacuum, first with carbon and then gold, to a total thickness of about 300 Å. They were scanned with a Stereoscan Cambridge S4-10. The beam potential was 20–30 kV and the current 180 μA [21].

Results

No Osmotic Gradient, No Hormone

Fig. 1 is a general view of the apical surface of the urinary bladder of the toad *Bufo marinus* under control conditions, that is, with neither osmotic gradient nor hormone. The picture is dominated by the presence of large polygonal cells corresponding to the granular cells described with light- and electron-microscopy [5]. The surface of the polygonal cells

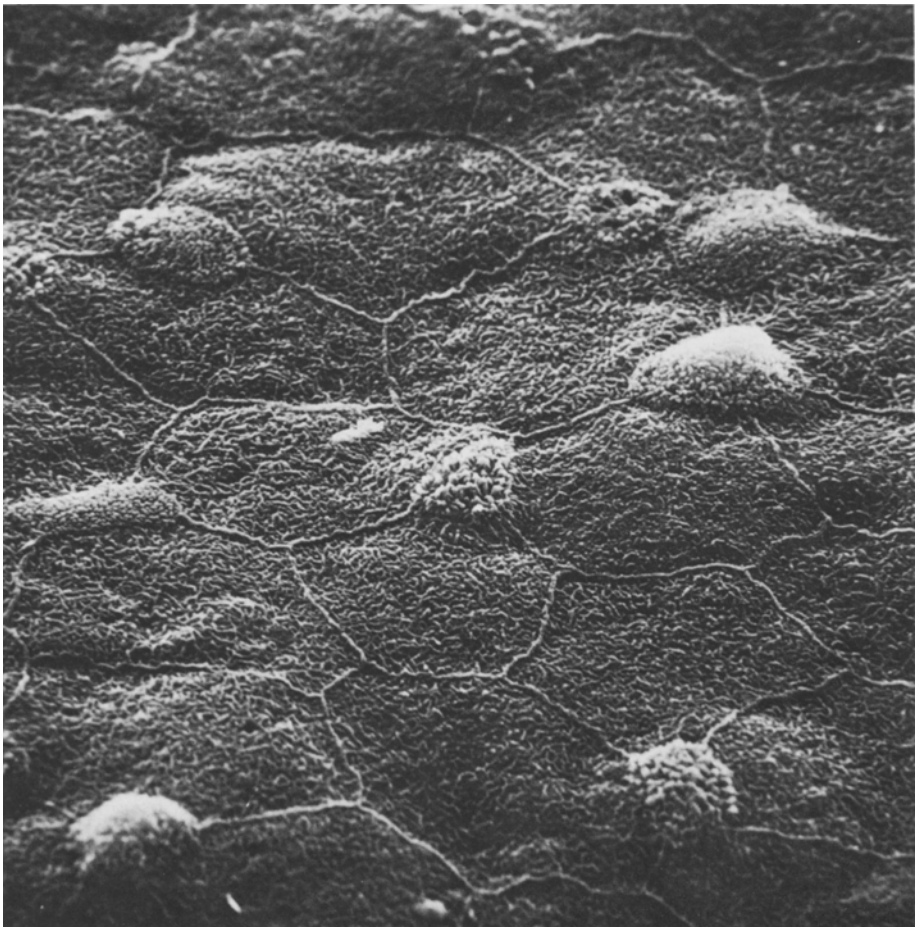

Fig. 1. General view of a urinary bladder of the toad *Bufo marinus* under control conditions (no gradient, no hormone). The large polygonal cells correspond to the granular cells of EM and the smaller, rounded cells to the mitochondria-rich cells (2,400 ×)

is covered with small, blunt microvilli, forming a ridge-like network. The cell borders are quite conspicuous and appear elevated with respect to the cell surface.

Interspersed with the polygonal cells, there are smaller, more rounded cells, with longer microvilli closely packed together. These cells correspond to the mitochondria-rich cells described in electron-microscopy [5]. A third cell type abutting to the apical surface is the mucus-producing goblet cell. In Fig. 2 a goblet cell is shown with a protruding mass of mucus coming out of the central pit. This cell type is also covered with long microvilli.

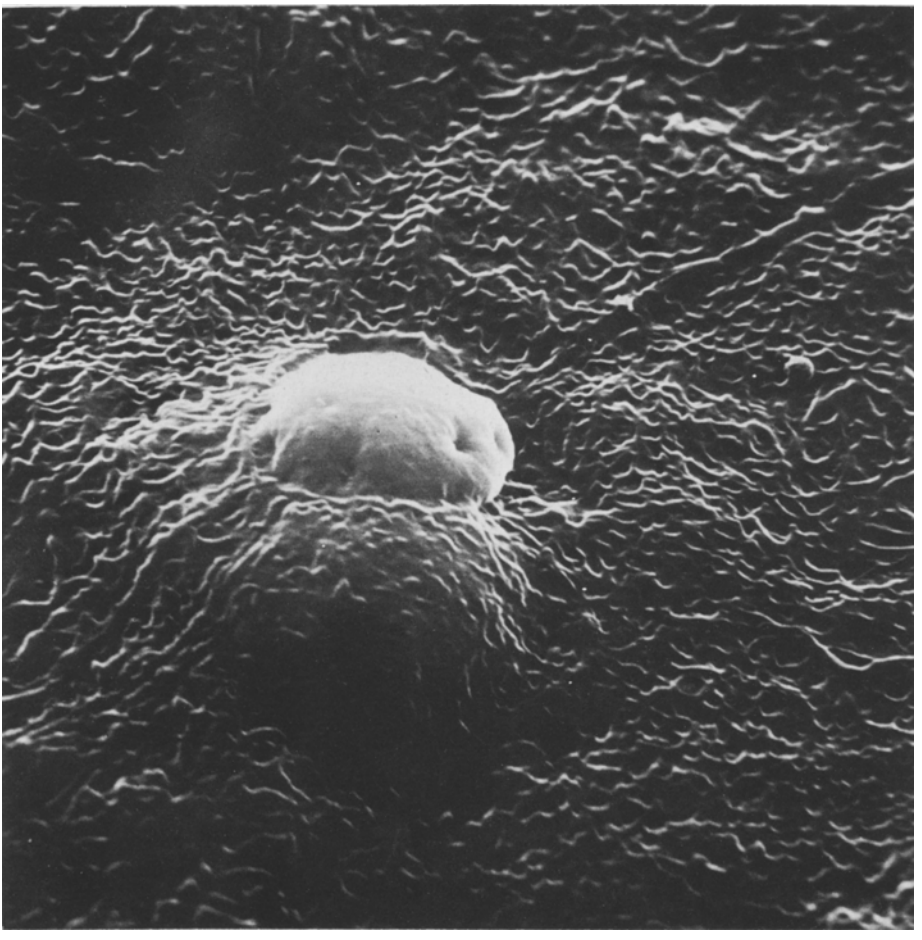

Fig. 2. A goblet cell, covered with microvilli and showing a protruding mass of mucus coming out of the central pit. Bladder under control conditions (6,000 ×)

Osmotic Gradient, No Hormone

Exposure of the urinary bladder to a large gradient obtained by dilution of the Ringer's solution bathing the mucosal surface did not alter appreciably the picture previously described. This is illustrated in Fig. 3 which shows the apical surface of a bladder exposed to an osmotic gradient 1:10. At higher magnification (Fig. 4), one sees the clear difference in the arrangement of the microvilli covering the surface of the granular cells and of those covering the mitochondria-rich cells. In Fig. 5 one sees the cell borders separating three contiguous granular cells. At this magnification, the cell borders appear somewhat irregular and present

Fig. 3. Urinary bladder exposed to an osmotic gradient 1:10 (mucosal side diluted). No conspicuous changes are seen when compared with Fig. 1 (2,400×)

discontinuities at some points. In between the ridges formed by the blunt microvilli, the cell surface shows a granular structure underneath which probably corresponds to the granules seen just beneath the apical membrane in electron-microscopy [3, 10, 11, 16].

Osmotic Gradient and Vasopressin

Exposure of toad bladder to the combination of an osmotic gradient and vasopressin produces striking physiological and morphological changes. The physiological response is well known and consists of an

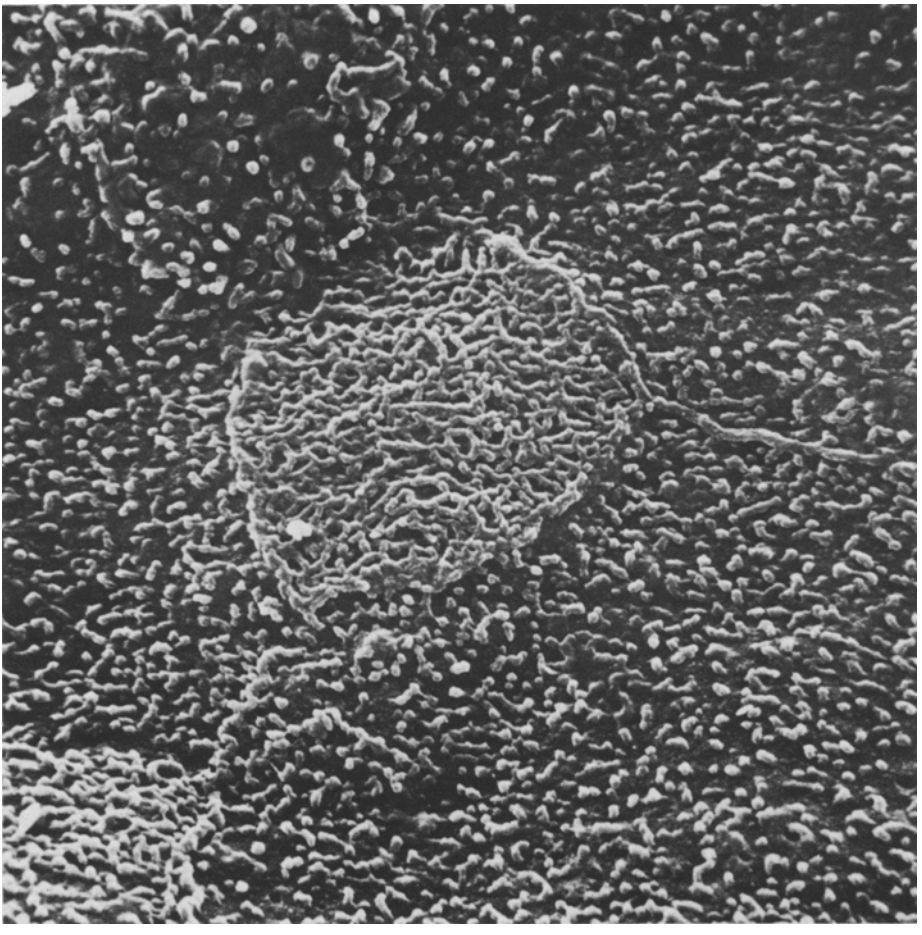

Fig. 4. Higher magnification of the bladder seen in Fig. 3. Note the different arrangement of the microvilli on the granular cells and on the mitochondria-rich cells (3,000 ×)

abrupt increase in water permeability. With SEM it is also possible to detect conspicuous morphological changes on the apical membrane of the epithelium during the hydrosmotic response.

Fig. 6 illustrates these morphological changes which occurred during the hydrosmotic flow induced by vasopressin in a bladder exposed to a 1:5 osmotic gradient. At the moment the bladder was fixed, the transepithelial water flux was 1.4 μliters min^{-1} cm^{-2}, a value close to the peak value of the response. The most prominent feature is a marked diminution

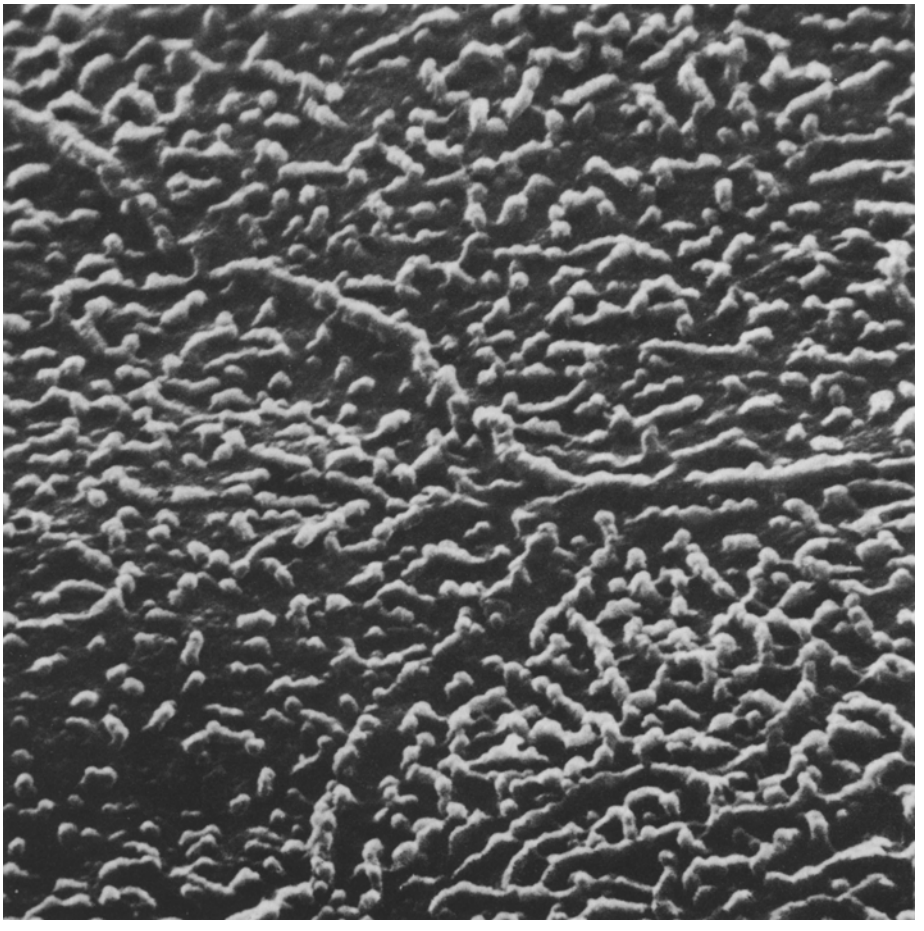

Fig. 5. Same bladder as in Figs. 3 and 4. The cell borders of three adjacent granular cells appear as the coalescence of two rows of microvilli (12,000 ×)

in the blunt microvilli covering the large polygonal cells. The degree of this reduction varies from one area to another, but it is not unusual to observe a total disappearance of the microvilli in large areas of the apical membrane. Fig. 7 shows another important aspect of these morphological changes, for while the granular cells show a reduction of microvilli (as already seen in Fig. 6), the structure of the mitochondria-rich cells seems totally unchanged. The long microvilli of the mitochondria-rich cell were examined at higher magnifications and in no instance were there signs of morphological alterations of the long microvilli (Fig. 8).

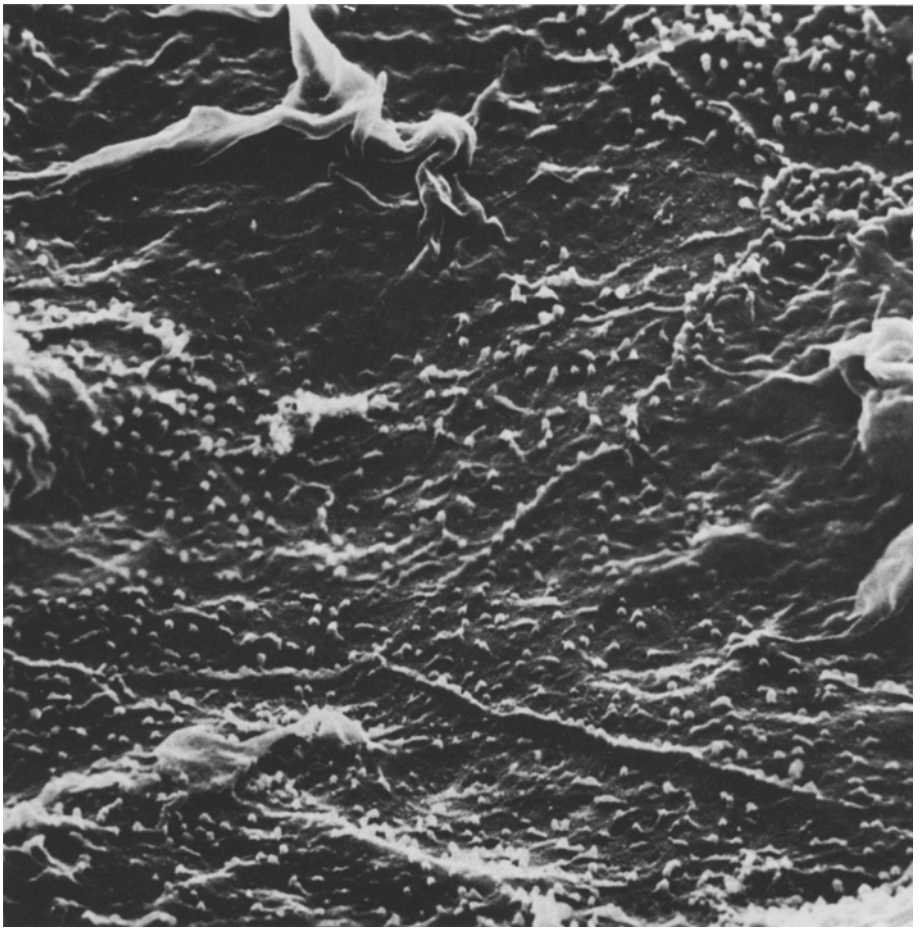

Fig. 6. Urinary bladder exposed to an osmotic gradient 1 : 5 and stimulated with vasopressin. Marked diminution of the blunt microvilli covering the granular cells (6,000 ×)

Osmotic Gradient and cAMP or Theophylline

Two other agents promoting a hydrosmotic response are the hormone messenger, cyclic AMP (cAMP), and the phosphodiesterase inhibitor, theophylline, and both were used to see if they also caused morphological changes.

The appearance at the SEM of the apical membrane of urinary bladders submitted to an osmotic gradient and exposed to cAMP was identical to that already described for vasopressin: loss of microvilli on the granular

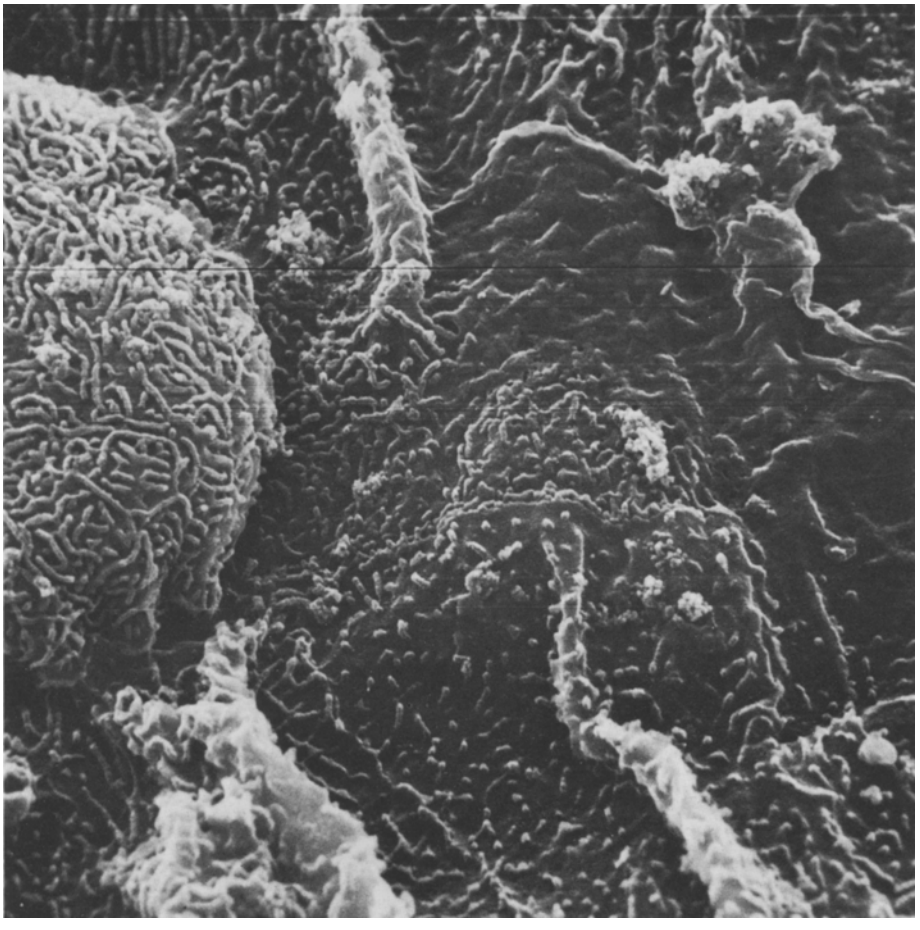

Fig. 7. Urinary bladder exposed to an osmotic gradient 1:5 and stimulated with vasopressin. The loss of blunt microvilli contrasts with the conservation of the microvilli covering a mitochondria-rich cell at the left side (3,000×)

cells while the structure appears unchanged on the mitochondria-rich cells.

Theophylline (10^{-2} M) was studied in a similar fashion with essentially the same effects. One observes the same conspicuous diminution of blunt microvilli although the pattern is somewhat more irregular, as can be appreciated in Fig. 9. One can see, side by side, areas with a relatively good conservation of blunt microvilli, while others are clearly deprived of microvilli. It is possible that this irregularity correlates with the magnitude of the water fluxes induced by theophylline, which were much lower than those induced by vasopressin or cAMP. Another important feature

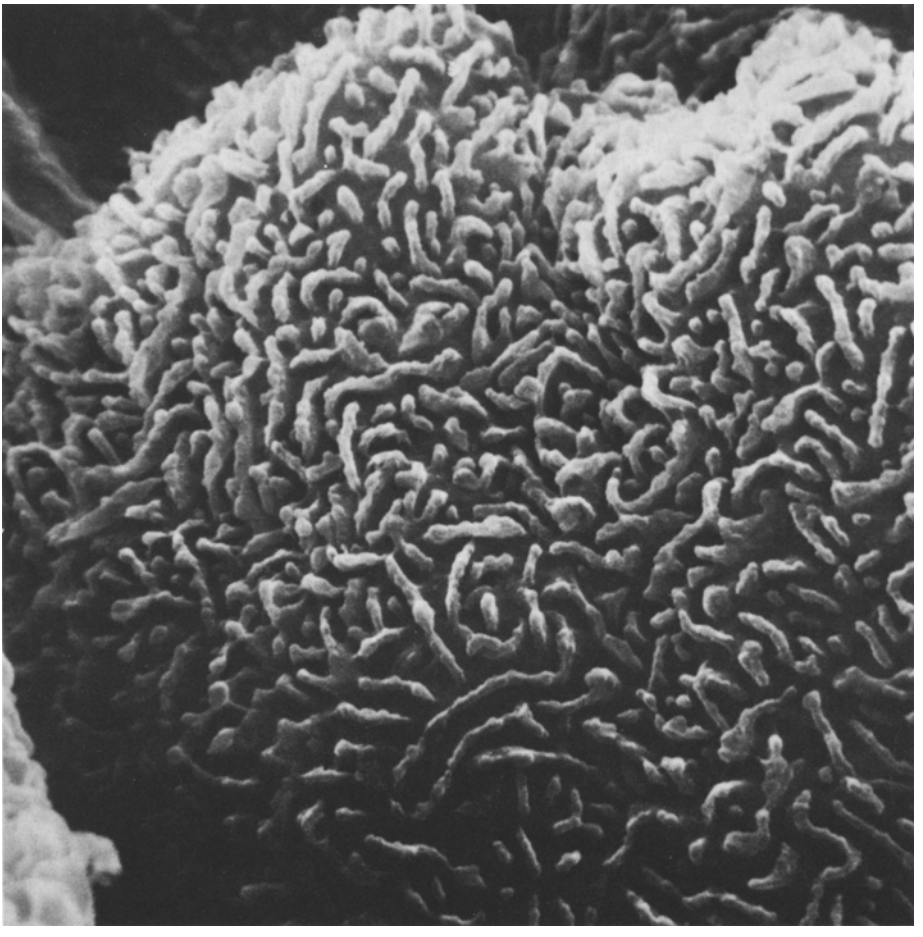

Fig. 8. Same bladder as in Fig. 7. View of a mitochondria-rich cell at a higher magnification (6,000 ×)

visible in Fig. 9 is the apparent preservation of the microvilli covering the goblet cells, during the hydrosmotic flow.

Vasopressin with No Osmotic Flow

A series of bladders was submitted to a protocol consisting of exposure to vasopressin in the absence of an osmotic gradient, an experimental condition during which there is no transepithelial net flux of water. As illustrated in Fig. 10, the apical membrane of the bladders shows no con-

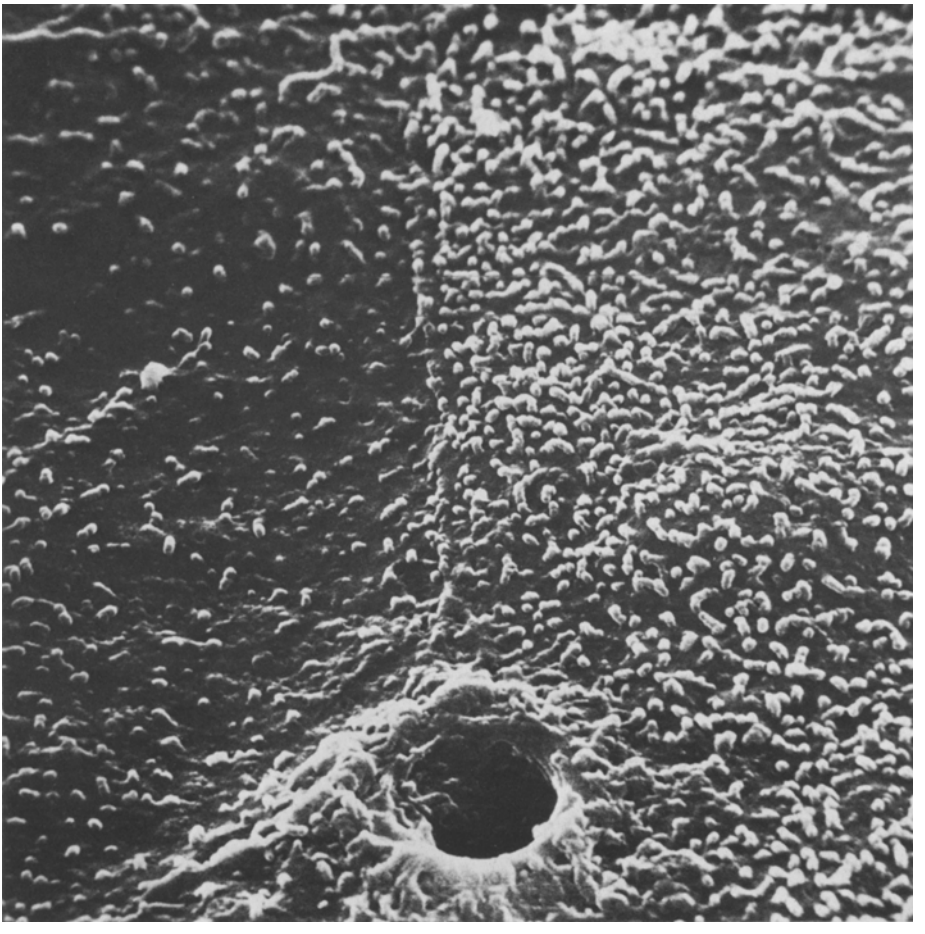

Fig. 9. Urinary bladder exposed to an osmotic gradient 1:10 and to theophylline (10^{-2} M). Alternation of areas with very few microvilli with areas of relatively well-preserved structure. At the bottom, the pit of a goblet cell (3,000×)

spicuous morphological changes when compared to control bladders and definitely does not show the disappearance of blunt microvilli which is regularly observed when vasopressin is given in the presence of an osmotic gradient.

Dilution of the Serosal Medium

Dilution of the medium bathing the serosal surface of toad bladder results also in morphological changes of the apical surface. In the final series of experiments (one of which is illustrated in Fig. 11) the serosal

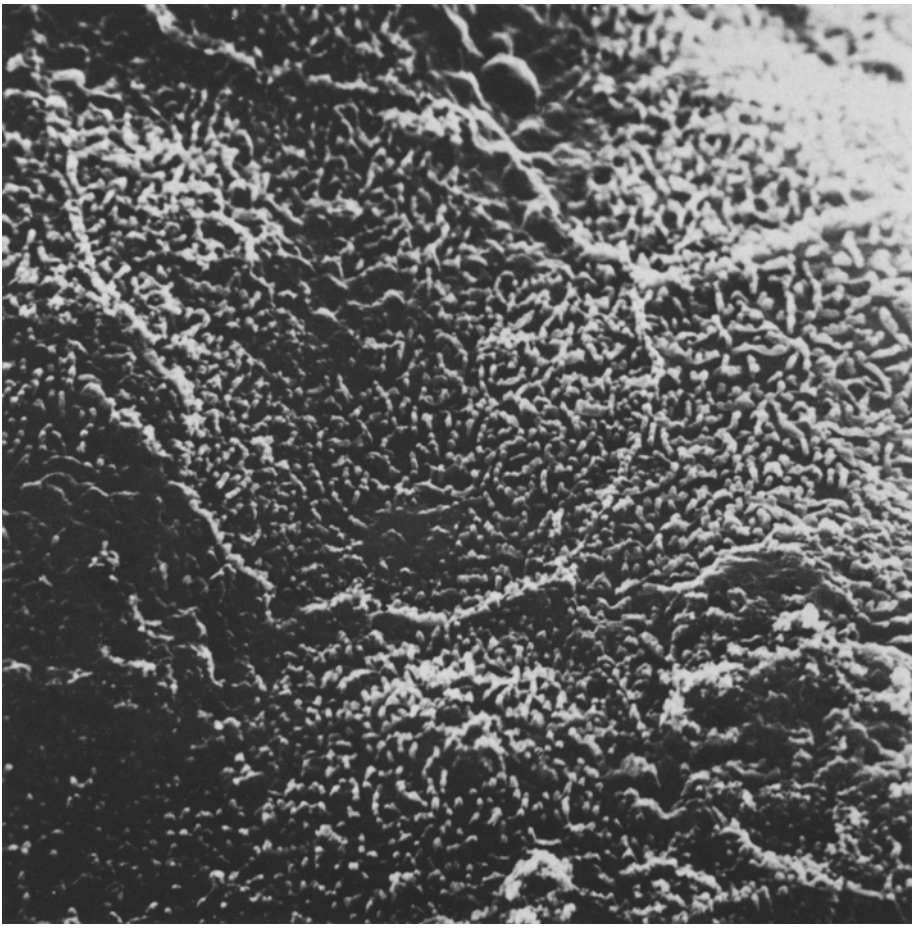

Fig. 10. Urinary bladder exposed to vasopressin in the absence of an osmotic gradient. No conspicuous changes are detected on the ridge-like network of granular cells (3,000×)

medium was diluted twofold. One observes the paucity of blunt microvilli as previously found in the presence of an osmotic flow. An additional feature is the appearance of blisters at the level of the cell borders. This is particularly evident in Fig. 12 where a row of blisters is clearly seen along the cell margins separating several granular cells.

Discussion

SEM appears to be a most valuable tool for studying the surface topography of epithelial membranes. We applied this technique to an

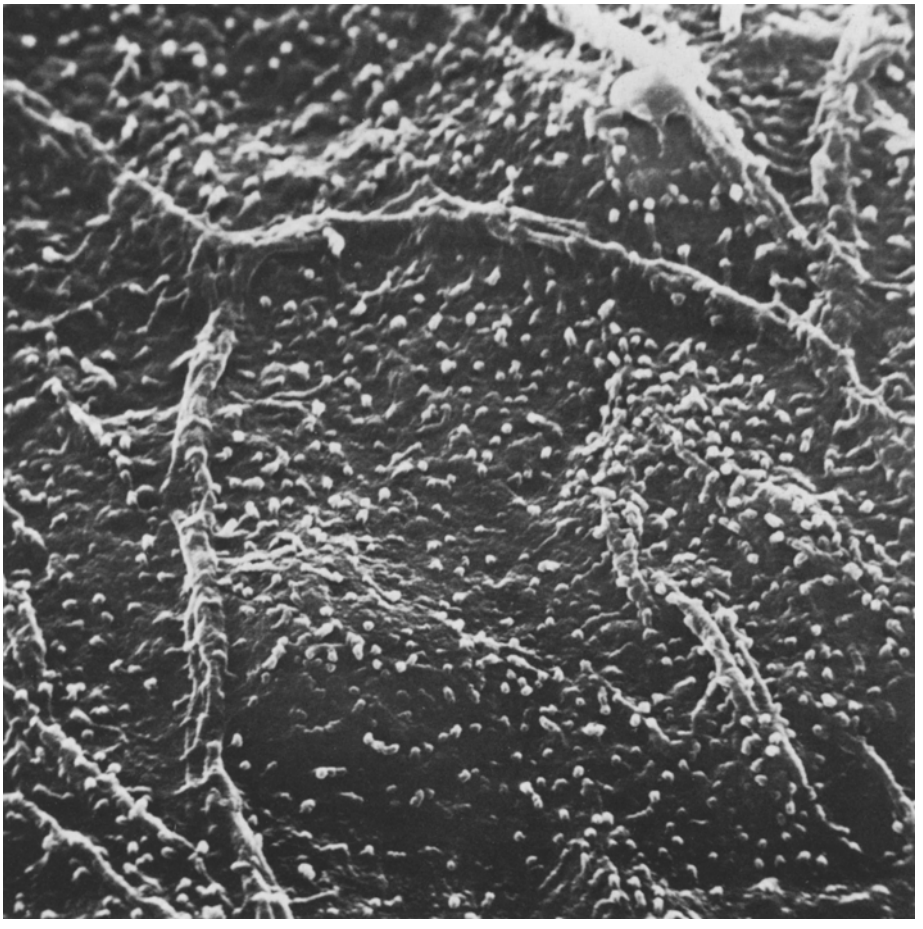

Fig. 11. Urinary bladder exposed to an osmotic gradient 2:1 (serosal side diluted twofold). Loss of blunt microvilli as seen in the presence of bulk flow of water induced by hydrosmotic agents. Presence of blisters on the cell borders (3,000×)

investigation of the hydrosmotic effect of vasopressin on the urinary bladder of the toad *Bufo marinus*. In the presence of an osmotic gradient, marked morphological changes were seen on the apical membrane of the epithelium during the hydrosmotic flow induced by the hormone, by cyclic AMP and by theophylline.

The apical surface of control bladders, exposed to normal Ringer's solutions on both the mucosal and the serosal sides, shows three types of cells, as depicted in Figs. 1 and 2. These findings confirm recent reports of SEM studies of toad bladder [5, 6, 11] and turtle bladder [18] and are consistent with the classical ultrastructural description of the amphibian bladder with transmission electron-microscopy [3, 10].

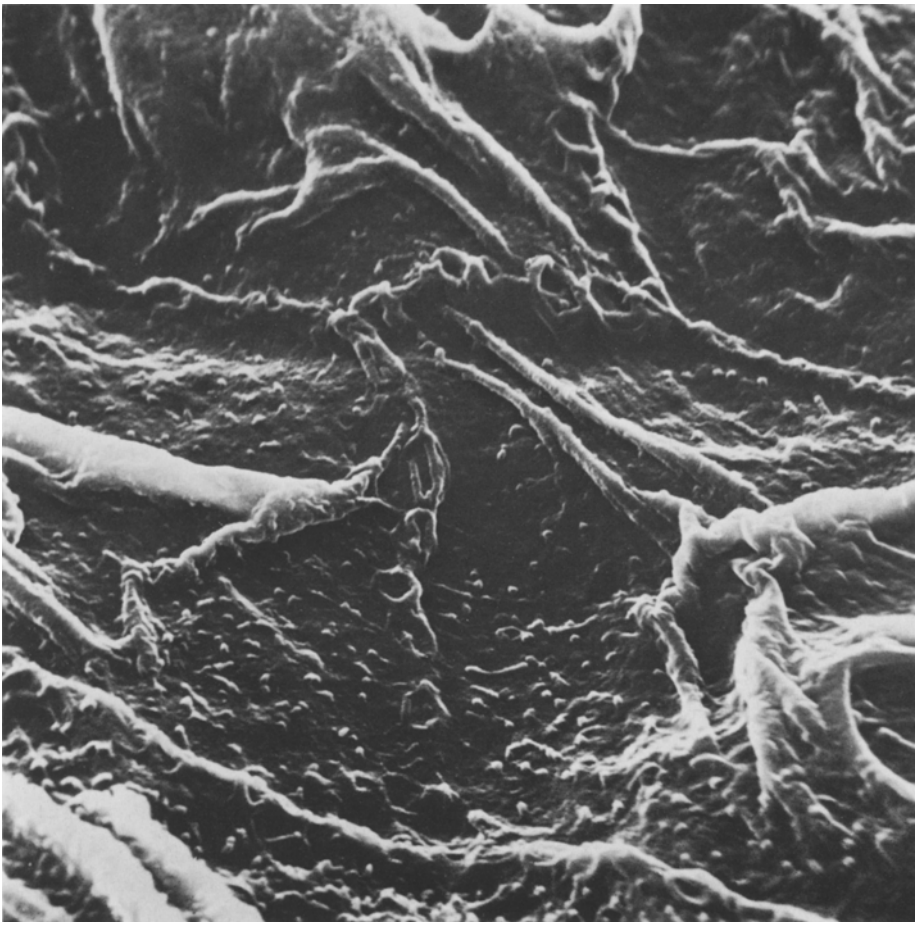

Fig. 12. Same bladder as in Fig. 11 showing a conspicuous row of blisters over the cell borders separating granular cells (3,000 ×)

All cell types abutting on the luminal surface are covered with microvilli, the nature and the arrangement of which vary from one cell type to another. The predominant, large, polygonal cells, corresponding to the granular cells of light- and electron-microscopy, are covered with blunt microvilli, forming a ridge-like network. The smaller, rounded cells corresponding to the mitochondria-rich cells, have much longer microvilli which are densely packed, as illustrated in Fig. 1. These cells have a higher secondary emission and are quite regularly distributed in a pattern of 4–5 granular cells surrounding each mitochondria-rich cell (Fig. 1). Finally, the goblet cells are also covered with long microvilli and, in addition, present a central pit which is sometimes filled with mucus (Fig. 2).

Dilution of the Ringer's solution bathing the mucosal side of the bladder did not change appreciably the surface of the apical membrane, as seen with SEM (Fig. 3). This applies to both the granular and the mitochondria-rich cells (Fig. 4). Such an observation is consistent with previous findings obtained with electron-microscopy in toad bladder [10] and in the cortical collecting duct of the rabbit [12]. It is also consistent with the physiological observations showing that, in the absence of neurohypophyseal hormones, toad bladder is quite impermeable to water, huge transepithelial osmotic gradients resulting only in minute water fluxes [7].

In contrast, a totally different picture is obtained when toad bladder is exposed to both an osmotic gradient and vasopressin. Two main observations were made: (a) the reduction of blunt microvilli on the polygonal (granular) cells; (b) the apparent conservation of the structure of the mitochondria-rich cells and of the goblet cells. Such findings are clearly illustrated in Figs. 5, 6 and 7.

Our results with SEM confirm and extend those of DiBona, Civan and Leaf [10], who, with electron-microscopy, were the first to provide morphological evidence of cellular specificity for the effect of vasopressin on water transport. Evidence has been obtained for cell specificity in other transport phenomena, using different techniques. For instance, recent data obtained with density gradient centrifugation suggest that the mitochondria-rich cells of the bladder might be the locus of H^+ secretion [20], as also seems to be the case in the turtle bladder [18]. As far as sodium transport is concerned, the partition of the cellular function must be reassessed in the light of recent data, but the possibility remains that the stimulation of sodium transport by neurohypophyseal hormones and by aldosterone may be exerted on different epithelial cell types [10].

There has been some controversy about the transepithelial pathway for bulk flow of water. Theoretical calculations showed that the paracellu-

lar pathway (or shunt pathway) through the tight junctions cannot be the primary route of water transport [4]. Our SEM results would seem to favor the transcellular pathway but do not necessarily exclude a concomitant paracellular pathway.

The results obtained with cAMP and theophylline are compatible with the concept that the hydrosmotic effect of vasopressin is mediated by an intracellular rise in cAMP. The biochemical steps distal to the generation of cAMP and leading to the change in permeability of the apical membrane are still unknown, but it should be pointed out that, in recent years, a number of alterations induced by neurohypophyseal hormones on the apical membrane of amphibian bladders have been reported. These include the correlation between pinocytosis and hydrosmosis [15], the hormone-stimulated exocytosis of the granules of the polygonal (granular) cells [16], changes in the negative surface charges [17] and changes in the distribution of membrane-associated particles, as revealed by the freeze-etching technique [2]. A related topic is the increased surface deformability of collecting duct cells induced by vasopressin and cyclic AMP [13].

The marked reduction of blunt microvilli in the presence of an osmotic gradient and vasopressin raises two questions: (a) are the morphological changes a direct consequence of the cellular effects of the hormone *per se;* or (b) are they a consequence of the entry of water into the cells and the resulting swelling. According to the first hypothesis one should see a paucity of blunt microvilli when bladders are exposed to vasopressin in the absence of an osmotic gradient. According to the second hypothesis, one should find the same morphological changes in the absence of vasopressin if the entry of water inside the cells is promoted by dilution of the serosal medium. Our results favor the second hypothesis and suggest that cell swelling is a prerequisite for the reduction of the blunt microvilli during hydrosmotic flow. These SEM observations perfectly match the results of the EM studies of DiBona *et al.* [10], but are in contradiction with those reported by Davis *et al.* [6], who also used SEM. At the present time, our data strongly suggest that in the absence of an osmotic gradient the blunt microvilli are well preserved when the bladder is exposed to vasopressin; in no instance did we see the marked surface changes occurring during the hydrosmotic response to vasopressin. However, it is more difficult to exclude completely the possibility that some minor changes in the density of the ridge-like network did occur when these bladders were exposed to vasopressin alone.

SEM reveals also that dilution of the serosal medium results in the formation of blisters at the level of the tight junctions (Figs. 11 and 12).

This was also observed with EM [8, 22] and more recently with the Nomarski interferential technique [9], whenever the mucosal medium is hypertonic with respect to the serosal medium.

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