Specific Vacuolation of Frog Urinary Bladder Granular Cell After ADH Stimulation of Water Transport

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Abstract. The present study deals with an analysis of specific traits of cell vacuolation induced by water flow and ADH. During incubation of frog urinary bladders in Ringer's solution diluted 2-fold, the water content of the bladder wall increased by an average of 19 %. In case of ADH-stimulated water flow the water content increased by an average of 15.7 %. Cell swelling induced by hypotonic conditions on the serosal side resulted in a drastic decrease of the response to the hydroosmotic action of ADH. Electron microscopy revealed significant differences between cells hydrated in the above conditions. Two-fold hypotonicity of the serosal solution caused a slight swelling of all types of cells accompanied by a narrowing of intercellular spaces. With ADH stimulation of water transport (at maximal water movement) granular cells were characterized by the presence of irregularly shaped giant vacuoles with processes. The limiting membranes of the vacuoles were closely connected with microtubules and microfilaments. The electron microscopic study of these cells by the freeze-substitution method revealed, in addition to giant vacuoles, a highly complex system of microtubules 35—40 nm in diameter. A morphological similarity was observed between the vacuolar systems of these granular cells and the contractile vacuole complex of protozoans. Possible mechanisms for the participation of giant vacuoles, electron-dense canaliculi, microtubules and microfilaments in transcellular water flow across epithelium are discussed.

Key words: Water transport — Frog urinary bladder — ADH — Vacuolation — Cell swelling

Introduction

The study of mechanisms of water transport regulation is one of the most challenging problems of general physiology. Progress has been made in the last few years in the investigation of the sequence of antidiuretic hormone (ADH) activated

intracellular processes leading to an increase of osmotic permeability (Handler and Orloff 1981); and inducing changes in the apical plasma membrane structure during water flow across the urinary bladder wall (Chevalier et al. 1974; Wade et al. 1981; DiBona 1983).

An increase in water absorption under the influence of ADH is accompanied by swelling of toad and frog urinary bladder cells (Peachey and Rasmussen 1961; Natochin et al. 1965) and an increase in the number of vacuoles (Mashansky et al. 1966; Masur et al. 1971; Davis et al. 1982). It is known that the basolateral membranes of the urinary bladder epithelial cells are more permeable to water than apical membranes. Permeability of the latter is regulated by ADH. This difference in membrane permeability made it possible to characterize experimentally specific traits of the swelling of cells and their vacuolation due to the increase of water flow in response to ADH, and the osmotic swelling of cells through basolateral membranes in the absence of ADH.

In the present study cell swelling induced by ADH and water absorption by a mucosal-to-serosal osmotic gradient was compared to that after soaking of bladders in a hypotonic Ringer's solution. A simultaneous investigation of the transport of water; tissue water content; and the ultrastructural study of the urinary bladder, enabled us to analyse specific features of cell vacuolation induced by water flow and by ADH.

Materials and Methods

Isolated urinary bladders of frog (*Rana temporaria*) were used for the experiment. The urinary bladder was filled in vivo and in situ with Ringer's solution (diluted 10-fold with distilled water). Isolated hemibladders were rinsed in vessels, containing aerated Ringer's solution. In special experiments the bladder was kept in Ringer's solution diluted 2; 4; and 10-fold with water or 2 and 4-fold with a free sodium chloride Ringer instead of water (for final osmolality of these solutions, see legend to Fig. 1). Osmotic permeability was estimated by changes in weight induced by osmotic water flow from the urinary bladder lumen to Ringer's solution (Natochin and Shakhmatova 1966). To measure the content of water and ions in tissue, pieces of the urinary bladder wall were lightly dried (i.e. excess fluid was removed), placed onto a pre-weighed quartz glass, weighed on a precision balance VLAO-100, dried down to a constant weight for 24 h in a thermostatic chamber at 105°C, and then weighed again. The dried sample was dissolved in concentrated HNO_3 at 80°C. Potassium and sodium concentrations were determined on a Zeiss III flame photometer using air-propane flame.

Electron microscopic examination was carried out by two methods. In one case the frog urinary bladder wall was fixed with a 2.5 % glutaraldehyde solution in cacodylate buffer, pH 7.2, and post-fixed with 1 % OsO_4 solution in the same buffer. Dehydration and embedding were performed using standard techniques. The osmotic concentration of fixative solutions was consistent with the osmolality of physiological saline solutions used in appropriate experiments. By means of the freeze-substitution method pieces of tissue were frozen rapidly on the smooth surface of a copper block cooled to the temperature of liquid nitrogen. Substitution of vitrified water was carried out in 1 % OsO_4 solution in acetone at 80°C over a period of 5 days. In this instance we succeed in preserving relationships between labile components of the cells. Unfortunately, good results were obtained only for a very narrow apical

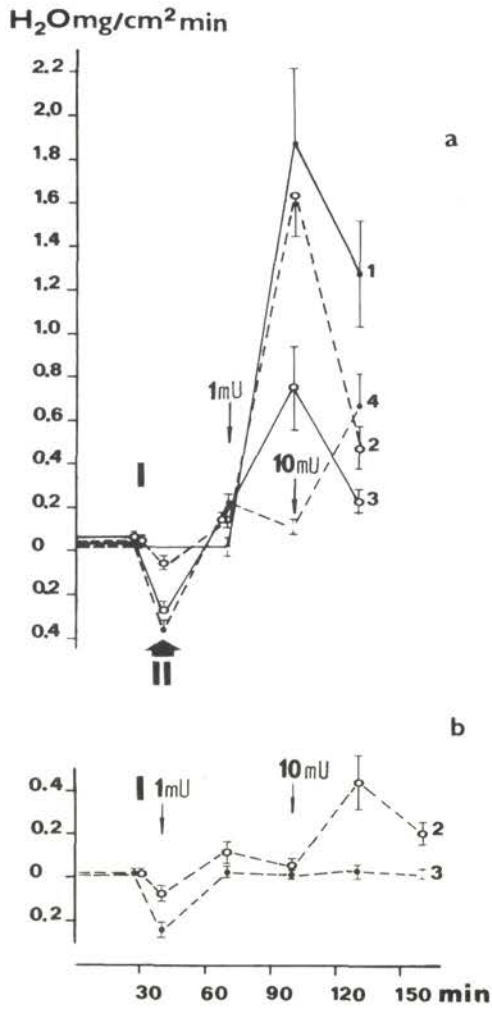

Fig. 1. The effect of ADH water transport across frog urinary bladder wall at various osmolalities of serosal Ringer's solution. Ordinate — water transport from urinary bladder lumen in mg/cm^2 per min; abscissa — the time of the experiment in min. In urinary bladders (in all experiments) mucosal Ringer's solution is diluted 10-fold with water — 22 mosm/kg H_2O; serosal Ringer's solution (in mosm/kg H_2O): 1—217, 2—110, 3—56, 4—22. **I**: change of serosal Ringer's solution for hypotonic solution of corresponding osmolality, **II**: restoration of initial osmolality of serosal Ringer's solution. Numerals near the arrow — final ADH concentration in mU/ml.

zone of the cell (2 or 3 μm from the frozen surface). In deeper layers crystallization of intracellular water was clearly visible.

In some experiments fixation was performed by the method of Komnick (1962) with the use of 2 % potassium pyroantimonate in 1 % OsO_4 solution in distilled water.

Results

Addition of ADH to the serosal Ringer's solution resulted in an increase in the water permeability of the urinary bladder. In the presence of an osmotic gradient there was a marked increase in water flow (Fig. 1*a*). In this case the water content of the bladder tissue ($n = 8$) increased from 3.74 ± 0.17 to 4.32 ± 0.22 kg H_2O/kg dry weight. The electron microscopic analysis revealed that in ADH-free Ringer's solution and in the presence of an osmotic gradient (hypotonic Ringer's solution at the mucosal surface) a well developed glycocalyx is clearly seen on apical plasma membranes of the epithelial cells (Fig. 2). Basolateral cell borders exhibited interdigitations forming a gap of 15 to 50 nm in width (Fig. 2*a*). With the presence of ADH a considerable swelling of presumably granular cells and the widening of intercellular spaces in the basolateral part of the epithelium was found to occur. Electron dense canaliculi localized, mostly in the apical region, and the presence of vacuoles were clearly evident in swollen granular cells.

To analyse these data a series of experiments was conducted. As outlined above, the apical membranes of urinary bladder epithelial cells are resistant to hypotonic solution while basolateral membranes of these cells are well permeable to water. Therefore, cell swelling in the presence of ADH was compared to that observed during transition from isotonic Ringer's solution to a hypotonic solution at the serosa. For this purpose the bladder wall was kept in Ringer's solution diluted with water for various time intervals (from 30 s to 10 min). The results obtained show that cell swelling is maximal at 2 min and that subsequent incubation in hypotonic medium does not significantly increase the water content of the bladder wall. Since in further experiments we intended to estimate the degree of swelling in solutions with drastically varying osmolality, the equilibrium time in hypotonic medium was increased to 10 min in all cases to ensure maximal cell swelling. In these experiments frog urinary bladders were placed in aerated Ringer's solution and after 60 min they were transferred for 10 min to a Ringer's solution diluted 2-, 4- and 10-fold respectively. The results obtained show that during incubation in Ringer's solution diluted 2-fold the water content in the bladder wall in separate experiments increased by 10—30 %, on average by 19 %. In response to ADH, with water flow across the bladder wall, cell swelling reached 15.7 % (Fig. 3). When the bladder wall was immersed in a Ringer's solution diluted 4-fold, swelling was greater than during ADH-induced water flow (Fig. 3).

Thus cell swelling occurs either after the stimulation of water flow by ADH or by immersion of the bladder into hypotonic solution. In the latter case, as a result of rapid cell swelling over a period of a few minutes, the bladder mass increases somewhat (Fig. 1*b*). The addition of 1 mU/ml ADH to Ringer's solution diluted

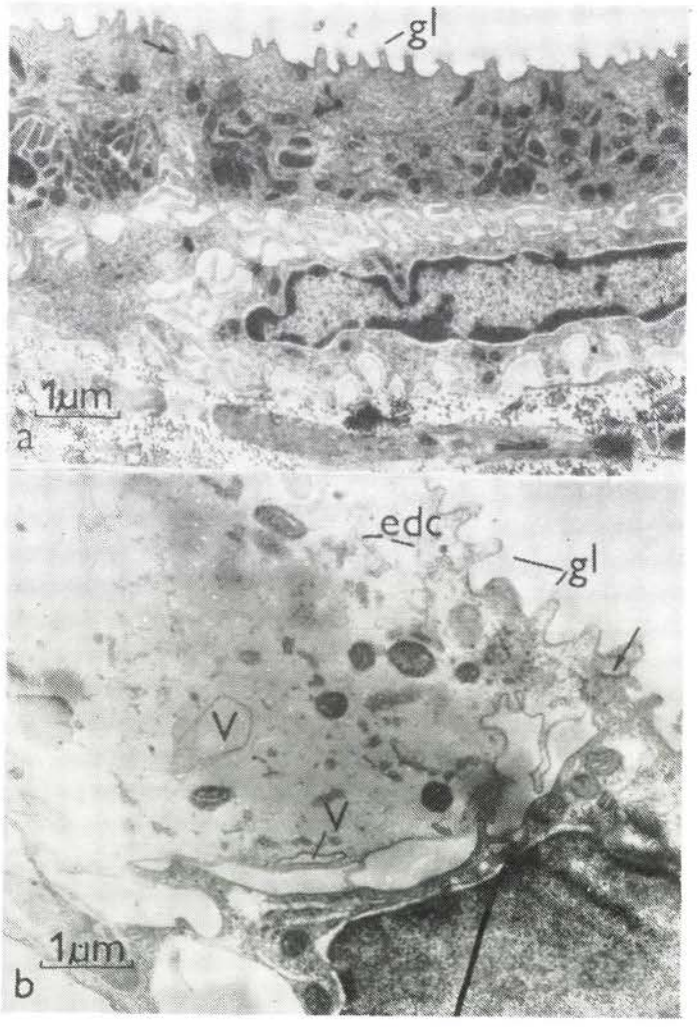

Fig. 2. Frog urinary bladder wall. *a* — control, ×14000, *b* — action of ADH, ×15000, gl — glycocalyx, edc — electron-dense canaliculi, v — vacuole, arrows — intercellular contacts.

2-fold failed to induce any further increase in water permeability, 10 mU/ml ADH was effective in inducing a slight but insignificant rise in water flow in the presence of an osmotic gradient (Fig. 1*b*). In the experiments with a Ringer's solution diluted 4-fold at the serosal surface even a higher increase in the ADH dose did not increase the permeability to water (Fig. 1*b*).

Nonsensitivity of cells to ADH might be associated either with changes in the functional state of the cell during swelling or with irreversible injury induced by

osmotic forces. To assess these possibilities the following experiments were performed: urinary bladders filled up with hypotonic Ringer's solution were transferred for 10 min to Ringer's diluted 2-, 4- and 10-fold and then returned to a standard Ringer's solution. The data show that in all cases the reaction to ADH was only partially restored, the degree of irreversible change being proportional to the degree of hypoosmotic injury of bladder cells (Fig. 1*a*). In experiments with the bladder wall immersed in a Ringer's solution diluted 2-fold, a small decrease in ADH response was observed 30 min after the addition of ADH when the maximum reaction took place; the permeability to water, however, diminished more sharply than in the control condition (Fig. 1*a*). After incubation in a Ringer's solution diluted 4-fold the ADH response was reduced throughout the whole period of hormone action. The effect was stronger when the urinary bladder was left in a maximally diluted Ringer's solution (22 mosm/kg H_2O). In this case the bladder cells did not react to 1 mU/ml ADH. With the increase of ADH concentration up to 10 mU/ml a weak although distinct reaction was visible. Consequently, cellular function was at least partly intact but cell reactivity was markedly reduced.

Dilution of Ringer's solution with water is associated with reduced osmolality of the solution and a decrease in the concentration of all ions. Experiments were conducted in which Ringer's solution was diluted 2 and 4-fold with sodium free Ringer's instead of water. We thus induced hypotonicity at calcium and potassium concentrations equal to those in standard Ringer's solution. The results show that during the first minutes in diluted Ringer's solution the cells of the urinary bladder swell and its mass increases (Fig. 1*a*). In the both cases the response of mucosal cells to the addition of 1 mU/ml ADH was poorly expressed. Thus it might be concluded that alteration in the reaction to ADH results from the decrease of the osmotic concentration in intercellular fluid and cell swelling, but not from the fall of the calcium and potassium concentration in this solution.

Measurements of the electrolyte content of the frog urinary bladder wall permit a finer analysis of the tissue response to hypoosmolality. When Ringer's solution was diluted with water and also in the case whereby in hypotonic solution, the potassium and calcium concentration was maintained at normal levels, the sodium and potassium content in the bladder tissue was reduced. Hence, the potassium content in tissue depends not only on the concentration of this ion in Ringer's solution but also on the concentration of sodium ions. It is known that hypokalemia and the decrease in the potassium content in cells diminish ADH reaction (Finn et al. 1966). Hypoosmolality exerts a similar effect but possibly more significant in magnitude (Fig. 3*a*).

The comparison of the ionic content of wet and dry substances shows that the total amount of sodium changes insignificantly while its concentration in tissue is reduced due to their cell swelling. The net amount of potassium in tissue decreases

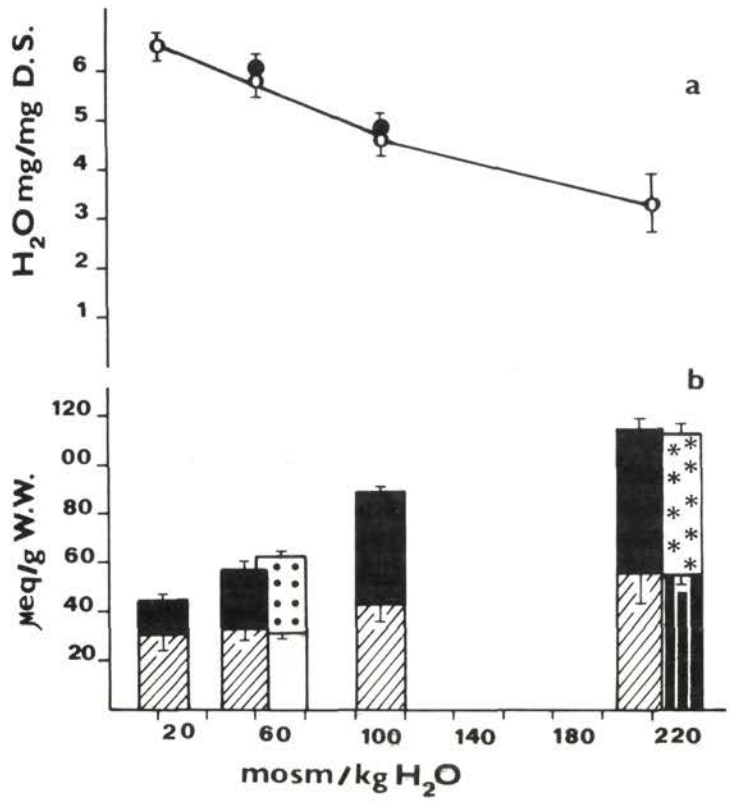

Fig. 3. Water and ionic content in urinary bladder tissue at the action of ADH and on change in the medium osmolality. Ordinate: *a* — water content in mg/mg dry weight, *b* — sodium and potassium contents in mmol/kg wet weight; abscissa — osmolality of serosal Ringer's solution mosm/kg H_2O. Arbitrary designations: ○ — H_2O; columns: obliquely stripped — Na, shaded — K — incubation in Ringer's solution diluted with water; ● — H_2O, empty — Na, dotted — K— the same upon dilution of Ringer's solution without NaCl, vertically bold stripped — Na, asterisked — K — action of ADH.

sharply in wet and dry substances, which is indicative of a true loss of potassium and not only the fall of its concentration as a consequence of tissue swelling. This may be accounted for by the inability of the cell to maintain potassium during swelling owing to a simultaneous action of hypoosmolality and low potassium concentration due to the diluted Ringer's solution. A comparison of the experiments in which the Ringer's solution was diluted with water with those employing sodium-free solution revealed that, in both cases, changes in the tissue sodium and potassium contents are similar in magnitude (Fig. 3*b*). Consequently, hypoosmolality produces a more marked and rapid effect than changes in the concentration of other ions (besides sodium) in physiological saline solution.

These results indicate that the decrease of reaction to ADH depends on cell hypoosmolality and swelling. Similar degrees of swelling of the urinary bladder induced by ADH or by soaking in a Ringer's solution diluted 2-fold was found to reduce ADH reaction more than that induced by water flow in the presence of ADH. This difference might be due to several reasons: the ability of ADH not only to increase permeability to water but to activate intracellular processes, protecting the cell from the destructive action of osmotic forces: various modes of action of osmotically free water depending on whether it flows through the apical or basolateral membrane of the cell; participation of different elements of the bladder wall during swelling in both experiments in the presence and the absence of ADH.

In studying mechanisms underlying different effects of cell swelling on the efficiency of ADH action, essential evidence may be provided by ultrastructural analysis of granular cells of the frog urinary bladder.

After chemical fixation (with glutaraldehyde) the cytoplasm of granular cells (Fig. 2) in control bladders at low water permeability contains granules of various shape and density, mitochondria, ribosomes, cytoskeleton elements (microtubules and microfilaments with respective diameters of 20—25 nm and 5—10 nm). Single microtubules are mainly oriented along the long axis of the cell, whereas microfilaments are distributed throughout the whole cytoplasm showing no preferable orientation. In vicinity of the apical plasma membrane they constitute a relatively dense layer. The vacuolar system of granular cells is represented by cisternae of the smooth and rough endoplasmic reticulum, cisternae of the Golgi apparatus and electron-dense canaliculi lying most commonly in the apical region of the cell. These canaliculi look like a branched anastomosing network, which is similar in some respects to elements of the tubulo-cisternal system previously described for frog skin, proposelly, participating in transcellular sodium transport (Møllgård and Rostgaard 1978).

After addition of ADH the cytoplasm was found to contain one or more giant vacuoles of irregular shape with processes (Fig. 2*b*, 4*a*, *b*, *c*). In different cells or occasionally in a single cell we have observed vacuoles varying in shape — from completely flattened to rounded. The membranes of the vacuoles are closely connected on the hyaloplasm side with microtubules and microfilaments. In tangential sections of these membranes connections of microfilaments with the membrane are visible (Fig. 4*a*, *c*). Not far from the vacuoles, elements of the Golgi apparatus and mitochondria are localized. In some cases connections between vacuole reservoirs and electron-dense canaliculi are evident.

The use of the Komnick technique for detecting some cations in the cell revealed an electron-dense precipitate on the extracellular surface of granular cells and at the periphery of large vacuoles. The precipitate is a layer about 0.12 μm thick, consisting of small granules. It must be stressed that with this technique it is practically impossible to reveal distinctly the limiting membrane of the vacuole.

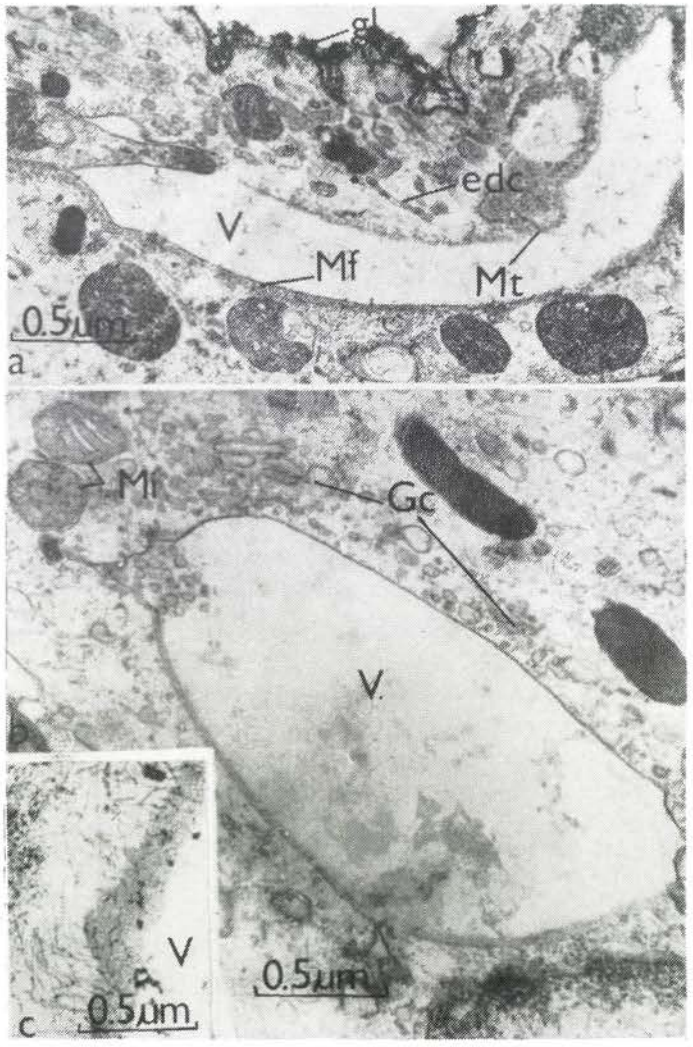

Fig. 4. Vacuoles of granular cells after the action of ADH. *a* — mitochondria (mi) localized in the vicinity of the vacuole (*v*). Microtubules (*mt*) and microfilaments (*mf*) are seen in immediate contact with the vacuole membrane. Glycocalyx is well developed, ×30000, *b* — elements of the Golgi complex (Gc) localized in the vicinity of the vacuole, ×50000, *c* — in lower tangentially sectioned parts of the vacuole membrane anchored microfilaments are seen (arrow), ×60000.

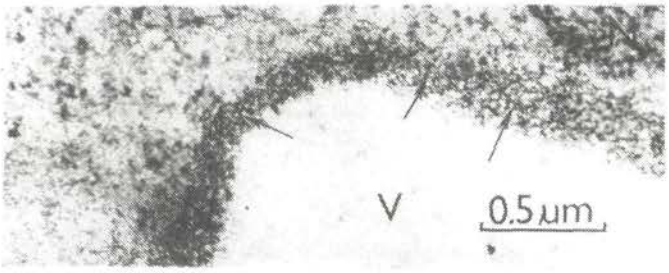

Fig. 7. Part of the vacuole (v) of granular cell at the action of ADH (fixation after Komnick). The precipitate consists of small granules (arrows); N — nucleus, ×53000.

Nevertheless due to the fact that this layer, along with pyroantimonate granules, contains microfilaments surrounding the vacuole it might be suggested that the precipitate is localized on its outer surface. Virtually, no precipitate is present in the vacuole cavity (Fig. 7).

The study of granular cells by the freeze-substitution method, adequate for detecting high-speed processes, also shows that at the action of ADH, cells possess a most complicated system of microtubules 33—40 nm in diameter along with large vacuoles. Occasionally elements of this system may be traced from surface regions of the cell to vacuoles (Fig. 5*a*, *b*, *c*). In the apical zone large aggregates of densely packed and differently oriented microtubules of the same diameter are distinctly visible. Apart from microtubules, electron-dense canaliculi occur from 40 to 80 nm in diameter, sometimes connected to the plasma membrane (Fig. 5*d*).

The detection of a great number of microtubules (single and organized in complexes) only by means of the freeze-substitution method points to the existence of high rate processes connected with their formation and destruction.

The picture is different in epithelial cells of urinary bladders immersed in Ringer's solution diluted 2-fold but not treated with ADH. In these experiments we observed a slight swelling of all kinds of cells accompanied by narrowing of intercellular gaps (Fig. 6). The cell organoids are swelling and a great number of small vacuoles come into view in the cytoplasm of these cells. In no case were the giant vacuoles described above found to occur. Consequently, giant vacuoles surrounded by microtubuli and microfilaments arise only in granular cells after stimulation of water flow with ADH which may be indicative of the participation of these vacuoles in water transport (Fig. 8).

Discussion

The results obtained suggest that swelling of cells and their hypotonicity reduce the effect of ADH. Apparent, morphological features of epithelial cell swelling are

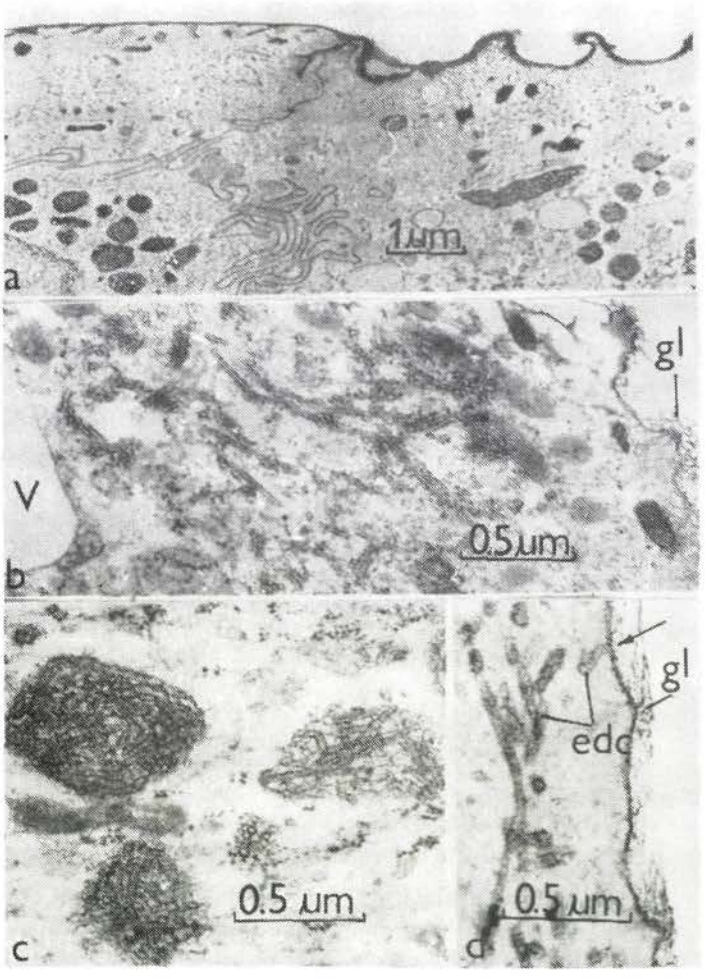

Fig. 5. Granular cells obtained by the freeze-substitution method. *a* — control, ×14000; action of ADH: *b* — complex of oriented microtubules is seen between the vacuole and apical plasma membrane, ×33000, *c* — aggregates of microtubules in cell apical zone, ×52000, *d* — electron-dense canaliculi are connected with apical plasma membrane (arrow), ×50000.

dependent on whether water flows from mucosa to serosa or into epithelial cells through basolateral membranes. The decrease in osmotic permeability of epithelium at cell hypoosmolality, and its increase at hyperosmolality, is regarded as a possible feedback mechanism providing regulation of cell permeability to water (Parisi et al. 1981).

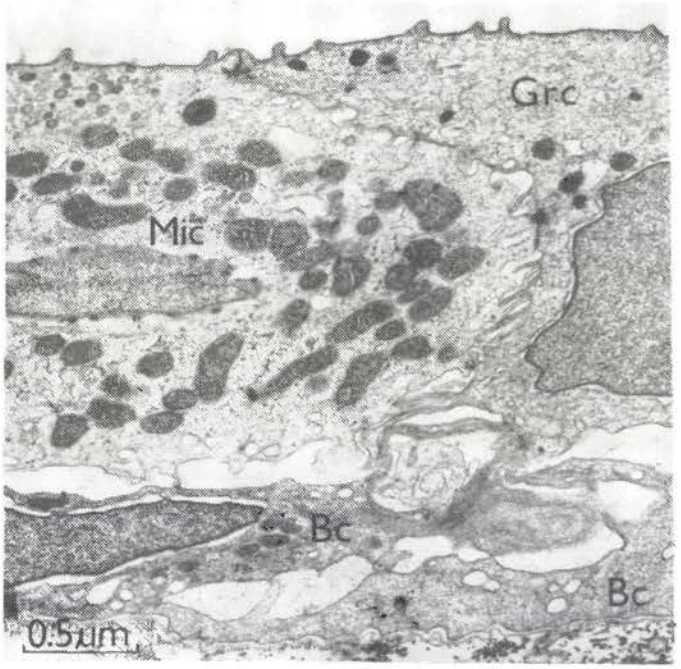

Fig. 6. Urinary bladder in hypotonic serosal Ringer's solution diluted 2-fold. Slight swelling of all types of cells (Mic — mitochondria-rich cell, Grc — granular cell, Bc — basal cell) and narrowing of intercellular gaps; a great number of small vacuoles in the cytoplasm.

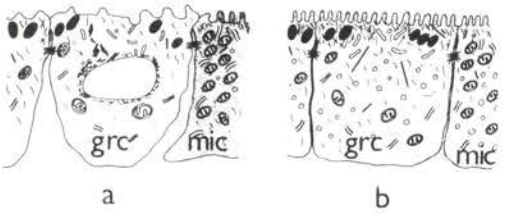

Fig. 8. Schematic representation of cell vacuolation. *a* — ADH action, *b* — hypotonic serosal solution. (For symbols see Fig. 6).

The question of the sequence of intracellular processes responsible for the increase in permeability to water and also for the role of subcellular structures in this process has been discussed extensively in the literature (Wade et al. 1981; Kachadorian and Levine 1982; Komissarchik et al. 1982; Snigirevskaya et al. 1982; Snigirevskaya 1983). The detection, in present study of microtubule

complexes beneath the apical plasmatic membrane in ADH stimulated bladders, along with available data on aggregation of intramembrane particles in the apical membrane (Chevalier et al. 1974; Kachadorian 1977; Bourguet et al. 1981) suggests a functional interrelation of these structural elements in terms of their participation in transepithelial water flow. This proposal is in agreement with findings showing that substances destroying microtubules and microfilaments inhibit the action of ADH (Taylor et al. 1973, 1978; Davis et al. 1974; Reaven et al. 1978). There is also evidence for the participation of the tubulo-cisternal system in transcellular transport of sodium and potassium ions (Møllgård and Rostgaard 1978; Forge 1982), and nonelectrolytes and proteins (Møllgård and Saunders 1977). There is evidence the participation of vesicular transport in permeability of epithelium for some anions and water (Oschman 1978; Davis et al. 1982; Andrews et al. 1983). The specialized vacuolar system we have observed in the frog bladder epithelium differs significantly from vesicular formations of other epithelia. Giant vacuoles surrounded by cytoskeleton elements are similar in structure to the contractile vacuoles of protozoans since they perform the osmoregulatory function in the cell (Patterson 1980; Snigirevskaya 1983). As in the case of contractile vacuoles in protozoans we have sometimes observed closely arranged mitochondria and giant vacuoles. The pattern of distribution of pyroantimonate precipitate over giant vacuoles of granular cells and contractile vacuoles of protozoans (Quader and van Kempen 1983) reveals a similarity in the localization of cations at the vacuole periphery. As demonstrated by X-ray microanalysis for protozoans, calcium is predominant in precipitate, surrounding the vacuole. Quader and van Kempen (1983) associate the presence of calcium ions around the vacuole with transport systems providing water flow to the contractile vacuole at the diastole stage. The possibility that such a mechanism is also inherent in the operation of giant vacuoles of granular cells may not be ruled out. At the same time, however, the concentration of calcium ions in this region may be connected with the functioning of contractile elements of the cytoskeleton providing for the activity of the vacuole.

Changes in the functional state of the cytoskeleton result in rearrangements of the cell vacuolar system. In epithelial cells of the toad urinary bladder treatment with ADH and cytochalasine B induced swelling followed by formation of giant vacuoles (David et al. 1974). The ultrastructure of these giant vacuoles differs however from that of the vacuoles described in this study. Their detection by means of the freeze-substitution method with ADH stimulated water flow is indicative of their role as a constant component of the cell response to hormone.

In considering water transport via vacuoles one of the most complex questions concerns the mechanism of hypotonisation of fluid in the vacuoles and the general functional significance of the vacuolar system. The latter appears to be a role in the transepithelial transfer of water or as an auxilliary apparatus for the restoration of cell water balance.

When there is appreciable water flow through epithelium, water may get to

parts of the cell which do not participate in its transfer, and, as a sequence, the cell swells and its activity deteriorates. The results obtained from the present experiments allowed the estimation of a quantitative measure of deterioration in ADH action during cell swelling. The vacuolar system we observed might form part of an intracellular mechanism, which prevents swelling of the cytoplasm and removes excess water from the cell. This is likely to account for a great structural similarity of the contractile vacuolar complex of protozoan cells and the vacuoles of bladder granular cells revealed during the action of ADH. In freshwater protozoans contractile vacuoles perform an osmoregulatory function; controlling the formation, from isoosmotic intracellular fluid, of hypotonic fluid and its successive excretion.

Several suggestions may be made as to how water enters the vacuolar cavity. By analogy with protozoans it may be assumed that water segregation is accomplished at the surface of tubular elements of the vacuolar system via a still uncertain mechanism (McKanna 1976; Patterson 1980), thereupon water is transferred to the main reservoir of the vacuole. This does not, however, preclude the existence of some different route. Among other things, this process might be manifested in terms of ionic sorption, dependent on the energy supply of adjacent mitochondria. Since the vacuole is not always surrounded by a great number of mitochondria, other mechanisms are possible. Rapid depolymerization of organic substances inside the vacuole (many of the vacuoles have a loose content of unknown composition) may lead to an increase of osmotic pressure in the vacuole and to an influx from the cytoplasm of water without dissolved substances which, in the final analysis, results in a filling of the vacuole. Subsequent polymerization of low molecular substances in the vacuole lumen would promote the formation of osmotically free water and its extrusion from the cell. Further experiments are necessary to assess the validity of the above hypotheses.

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