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Action of Vasopressin, Ouabain, and Cyanide on the Volume of Isolated Toad Bladder Epithelial Cells

Patrick Eggena

Department of Physiology and Biophysics, Mount Sinai Medical and Graduate Schools of the City University of New York, New York 10029

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Summary. Toad bladder epithelial cells were isolated under mild conditions in a calcium-free medium; they were found to exclude trypan blue, to consume oxygen, and to respond to vasopressin with an increased rate of oxygen consumption. Since isolated toad bladder epithelial cells are mostly spherical in shape, the cell diameter can be accurately measured with an ocular micrometer of an inverted microscope. Epithelial cells swelled by $29 \pm 3\%$ in the presence of KCN. This cyanide-induced swelling of cells was prevented by amiloride or, alternatively, by replacing NaCl by equiosmotic amounts of mannitol in the Ringer's fluid. Cells incubated in the presence of vasopressin swelled by $10 \pm 2\%$. Vasopressin and KCN acted synergistically in enhancing cell volume. Ouabain caused cells to swell by $9 \pm 2\%$, and this effect was not additive to the swelling seen with vasopressin. These observations are in accord with the theory of Leaf and his associates, that the predominant effect of vasopressin is to enhance sodium entry into the transporting epithelial cells of the toad urinary bladder.

Since the early report by Leaf *et al.* [20], that the isolated urinary bladder of the toad transports sodium from lumen to interstitium, this tissue has been used extensively in studies aimed at defining the cellular mechanism of action of vasopression [21] and aldosterone [4, 7]. There seems to be general agreement that sodium moves across the apical plasma membrane from lumen to cytoplasm by a passive process of facilitated diffusion, and sodium is then extruded from the cell against an electrochemical gradient across the basal-lateral plasma membrane [13]. Some investigators have suggested that vasopressin affects only the sodium entry step into the cell [3, 13, 21] while others [10, 12, 18] have favored an additional action of vasopressin on the active extrusion of sodium across the basal-lateral membrane or on the supply of energy to the sodium pump. In an effort to resolve this question regarding the site of action of vasopressin, Macknight *et al.* [24] measured the

intracellular sodium pool with isotopic sodium, and found that this pool increased in the presence of vasopressin. This observation was confirmed by Handler *et al.* [16] using different techniques for measuring the sodium pool of isolated toad bladder epithelial cells. Moreover, they also observed that aldosterone increased intracellular sodium, but they point out this change in cell sodium reflects the predominant effect of the hormone and does not rule out an additional effect on sodium transport across the basal-lateral membrane. Lipton and Edelman [22] analyzed the sodium pool of isolated toad bladder epithelial cells by atomic absorption spectrometry and measured water content of cells from wet and dry weight determinations. They were unable to repeat the observations of the above experimenters [16, 24] and they concluded that both vasopressin and aldosterone have dual sites of action on the sodium entry and exit steps.

The present study was undertaken to see whether or not isolated toad bladder epithelial cells swell in the presence of vasopressin as they should if the hormone facilitates sodium entry into the cell at a faster rate than the sodium can be pumped out. We have found previously [9] that cells isolated under mild conditions assume a nearly spherical shape, so that it is possible to measure the diameter of live cells in suspension with the micrometer of an inverted microscope. Using this direct microscopic approach in the study of cell hydration, the reports by others [16, 24] that vasopressin increases the cell water content have been confirmed.

Materials and Methods

Toads of the species *Bufo marinus* (subspecies from the Dominican Republic) were purchased from National Reagents Inc., Bridgeport, Conn., between July and October, and they were kept on moist peat moss at room temperature. Toads were doubly pithed, their urinary bladders were isolated, and the hemibladders were then suspended with the mucosa on the inside on a cannula. Hemibladders were filled with a balanced salt solution which was calcium-free. This solution was made fresh daily and it had the following composition (in mM): NaCl, 137; glucose, 5.6; $KHCO_3$, 4.2; and phenol red, 0.014. After the hemibladders had been incubated in this solution for 1 hr at room temperature, they were emptied and gently massaged between thumb and forefinger to loosen the attachments of the epithelial cells with one another and the basement membrane. Isolated epithelial cells were collected with a syringe through the cannula and resuspended in "Ringer's fluid". This Ringer's fluid was identical in composition to the solution detailed above used for isolating cells with the exception that 1 mM $CaCl_2$ and 1 mM $MgCl_2$ was now added. The osmolality of the Ringer's fluid as measured with a Fiske Osmometer was

270 mOsm/kg H_2O[1] and its pH was 7.4. Cells were equilibrated in Ringer's fluid for 1 hr, and then incubated at room temperature for another hour in the experimental media detailed in the Results.

Epithelial cells were sized by transferring a 500-λ aliquot of cell suspension to the well of a petri dish (type NUM-PD, Unitron) and viewing live cells with the oil-immersion lens (1000× magnification) of a Unitron Series N inverted microscope. With the aid of an ocular micrometer the diameter of all cells within a given field was measured, provided the cells were both round and single. One hundred cells were sized in each sample within a period of approximately 10 min. During this time interval no change in the temperature of the solution under the microscope could be detected, although the temperature did rise by 1 °C after a period of 60 min under the scope. Cell diameter could be measured with an accuracy of ± 1 μ under these conditons. Since the mean cell volume was found to vary from one toad to another, the same suspension of cells was always used when a comparison was to be made between different experimental conditions. In order to avoid observer bias, all specimens were coded by one person while another measured cell diameters under "blind" conditions.

Oxygen consumption was measured with a YSI Model 53 Oxygen Monitor, and cells were counted with a Neubauer chamber under a microscope. Pitressin (Parke-Davis) was used as a vasopressin source in a final concentration of 200 mU/ml. Ouabain (Sigma) and ethacrynic acid (Merck, Sharp & Dohme) were used in a concentration of 1 mM. Amiloride was kindly supplied by Merck, Sharp & Dohme and used in a concentration of 0.01 mM.

Results

Cells isolated under the conditions detailed above appeared to be viable in the sense that they excluded trypan blue, consumed oxygen, and responded to vasopressin. In one study on these cells the oxygen consumption was found to be 3.78×10^{-6} μliters O_2/hr/cell[2] in the absence and 5.67×10^{-6} μliters O_2/hr/cell in the presence of vasopressin. The epithelial cells of the toad bladder mucosa vary markedly in size as can be seen from the histogram in Fig. 1. In this study 700 cells taken from seven different toads were found to range in size from 905 to 6371 μ^3. Nevertheless, different aliquots taken from the same cell suspension could be sized with a consistency of $\pm 5\%$ (Table 1).

To learn about the relationship between sodium pump activity and epithelial cell volume several agents known to inhibit the short-circuit

1 This solution is slightly hypertonic compared to the amphibian Ringer's employed by most investigators. A hypertonic solution was chosen here to increase the sensitivity of cells to vasopressin [9] on the one hand, and on the other hand to increase the accuracy of measurement of cell diameter by magnifying the fractional increase in cell diameter for a given change in cell volume.

2 The protein content of isolated toad bladder epithelial cells was found to be $293 \pm 6 \times 10^{-3}$ μg/cell. Therefore, the oxygen consumption of these cells also corresponds to 0.013 and 0.019×10^{-3} μliter O_2/hr/μg protein in the absence and presence of vasopressin.

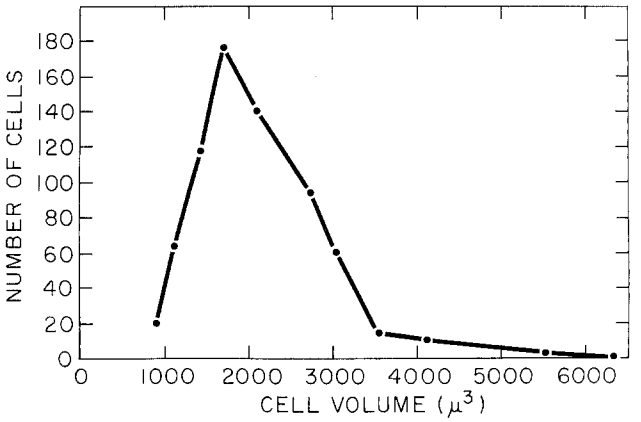

Fig. 1. Histogram of toad bladder epithelial cells equilibrated in 270 mOsm/kg H_2O medium

Table 1. Consistency of the method used for sizing epithelial cells with a microscope

Sample No.	Cell diameter (μ)	Cell volume (μ^3)	Percent of control
1	15.06±0.12 (SEM)	1793	103.9
2	14.87±0.11	1725	100.0
3	15.08±0.11	1798	104.2
4	15.00±0.13	1771	102.7
5	15.10±0.19	1808	104.8

Epithelial cells were isolated from a single toad bladder following exposure to a calcium-free medium, and they were subsequently incubated in calcium-containing Ringer's fluid for 60 min. Five 500-λ aliquots of the cell suspension were placed in the well of a petri dish and 100 cells per sample were sized within a period of 10 min. All but sample no. 4 were sized by the same observer.

current across the isolated toad bladder were tested. As is shown in Table 2, cells swelled by 29±3% (SEM) within a period of 60 min when KCN was present in the medium. Ouabain and ethacrynic acid also caused cell swelling, but only by 9±2% and 5±1%, respectively. As is shown in Table 3, the cell swelling normally seen following exposure to KCN is completely prevented when amiloride is added to the medium. Similarly, substituting all the NaCl in the medium by equiosmotic amounts of mannitol prevents the KCN-induced swelling of epithelial cells. Indeed, cells were found to shrink by about 9% when mannitol replaced NaCl in Ringer's fluid.

In the presence of vasopressin toad bladder epithelial cells were found to swell by 10±2% (SEM), and this effect of vasopressin appeared to be greater in the presence of KCN (Table 4). Such a synergistic effect

Table 2. Effect of potassium cyanide, ouabain, and ethacrynic acid on cell volume

Incubation medium	Cell volume	
	μ^3	Percent of control
Ringer's (control)	2186	100
Ringer's plus 1 mM KCN	2836	129 ± 3 $p < 0.001$ [16]
Ringer's (control)	2116	100
Ringer's plus 1 mM ouabain	2313	109 ± 2 $p < 0.001$ [11]
Ringer's (control)	2505	100
Ringer's plus 1 mM ethacrynic acid	2613	105 ± 1 $p < 0.02$ [3]

Cells were isolated from a single hemibladder and incubated for 60 min in the media detailed in the Table. One hundred cells were sized under control and experimental conditions and the average volume was computed. This procedure was repeated several times on the number of toads given in parentheses and the mean volume recorded in the Table. The data obtained in each hemibladder were also expressed as a percentage of the control value, and the mean value with the standard error of the mean for experiments on several toads is listed in the Table.

Table 3. Effects of amiloride and mannitol on cyanide-induced epithelial cell swelling

Incubation medium	Cell volume	
	μ^3	Percent of control
NaCl-Ringer's (control)	2239	100
NaCl-Ringer's plus 1 mM KCN	2754	124 ± 3 $p < 0.001$ [5]
NaCl-Ringer's plus 1 mM KCN plus 1×10^{-5} M amiloride	2261	103 ± 3 $p < 0.01$ [5]
NaCl-Ringer's (control)	2314	100
Mannitol-Ringer's	2101	91 ± 2 $p < 0.01$ [3]
Mannitol-Ringer's plus 1 mM KCN	2008	88 ± 2 $p > 0.2$ [3]

Legend as in Table 2.

Table 4. Effect of vasopressin on cell volume in the presence and absence of cyanide

Incubation medium	Cell volume	
	μ^3	Percent of control
Ringer's (control)	2003	100
Ringer's plus vasopressin	2207	110 ± 2 $p < 0.001$ [9]
Ringer's plus KCN	2610	130 ± 6 $p < 0.001$ [9]
Ringer's plus KCN plus vasopressin	2968	147 ± 7 $p < 0.001$ [9]

Legend as in Table 2.

Table 5. Effect of vasopressin on cell volume in the presence and absence of ouabain

Incubation medium	Cell volume	
	μ^3	Percent of control
Ringer's (control)	1939	100
Ringer's plus vasopressin	2156	111 ± 3 $p < 0.001$ [6]
Ringer's plus ouabain	2077	107 ± 2 $p < 0.001$ [6]
Ringer's plus ouabain plus vasopressin	2133	110 ± 4 $p < 0.001$ [6]

Legend as in Table 2.

was not observed between ouabain and vasopressin; indeed the swelling observed with each agent individually was not additive when both were present in the medium together (Table 5).

Discussion

Toad bladder epithelial cells isolated following a 1-hr period of exposure to a calcium-magnesium-free balanced salt solution have been found to take on the shape of a sphere, so that cell diameters can be measured accurately with an ocular micrometer while viewing live cells under an inverted microscope. Cells isolated in this manner appeared to be viable in that they were found to exclude trypan blue, to consume oxygen, and to respond to vasopressin with a 1.5-fold increase in oxygen consumption. We had observed earlier [8] a twofold increase in oxygen consumption and a 3.5-fold increase in intracellular cyclic AMP [9] on cells isolated under similar conditions from different batches of toads. It is well known from studies of others [6, 14, 21, 25, 26] that vasopressin increases the oxygen consumption of the isolated toad urinary bladder.

Ouabain, in concentrations known to be effective in blocking the short-circuit current across the toad bladder epithelium [17], triggered a 9% increase in cell volume in the present study. This finding is at variance with the observations of others [16, 22, 23] that isolated toad bladder cells gain sodium and lose potassium without any change in hydration in the presence of ouabain. In those studies tissue hydration was determined from a comparison of wet and dry weights with the use of inulin as an extracellular marker. In the present study changes in cell volume were measured microscopically.

It has been suggested [15] that volume regulation in proximal tubular epithelial cells is achieved by a ouabain-insensitive sodium pump which is inhibitable by ethacrynic acid. When ethacrynic acid was tested in the present study on toad bladder cells in concentrations known to be effective in inhibiting the short-circuit current across the intact bladder [2], only minimal, i.e. 5%, swelling of isolated cells was observed. In contrast, in the presence of KCN cells were found to swell by 29% indicating that aerobic metabolism is required to regulate cell volume. It is quite possible [22] that energy metabolism affects membrane permeability in addition to regulating the sodium pump, so that the rather marked effect on cell volume by cyanide could be due to increased entry of sodium chloride into the cell coupled with inhibition of the sodium extrusion mechanism(s). Cell swelling under anaerobic conditions or in the presence of metabolic inhibitors is a well-known phenomenon seen in many tissues. Leaf [19] has suggested that sodium extrusion from cells normally opposes the colloid osmotic force generated by impermeant intracellular macromolecules, so that sodium, chloride, and water move into cells when active extrusion is prevented by metabolic inhibitors. This hypothesis is consistent with the observation in the present study that swelling of isolated toad bladder epithelial cells is completely prevented when the NaCl of the incubation medium is replaced by equiosmotic concentrations of mannitol. Furthermore, in the presence of amiloride the cyanide-induced swelling was completely prevented, suggesting that sodium movement into the cell was blocked. Amiloride is known to inhibit the short-circuit current across the intact epithelium primarily by inhibiting sodium entry into the cell across its mucosal boundary [1, 5], although the possibility of an additional inhibitory effect on the sodium pump itself has not been ruled out as yet [11].

The degree of cell swelling observed in the present study with vasopressin was quantitatively very similar to the increase in cell water content measured by Macknight *et al.* [24] and Handler *et al.* [16] employing different techniques. Although this finding quite clearly indicates that vasopressin increases salt movement into toad bladder cells, an additional stimulatory effect on the sodium pump itself cannot be excluded. Indeed, the increment in cell volume resulting from vasopressin was considerably greater in the presence of cyanide than in its absence. This is consistent with an increased rate of extrusion of sodium from cells stimulated by vasopressin and the absence or reduction of such extrusion when metabolism is poisoned by cyanide. In contrast, ouabain did not increase the vasopressin-induced increment in cell swelling, which one might have

expected if ouabain inhibits the sodium pump. However, Finn has recently pointed out [11] that the action of ouabain is more complex than was formerly thought and he has presented evidence that this agent not only inhibits sodium extrusion but also inhibits sodium entry into the transport pool of the toad urinary bladder.

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