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Effects of Vasopressin on the Water and Ionic Composition of Toad Bladder Epithelial Cells

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Summary. Isolated sheets of epithelial cells as well as epithelial cells scraped from paired hemibladders mounted in chambers both showed significant increases in water, sodium and chloride contents after exposure to vasopressin (100 mU/ml), without any change in potassium content. In the isolated cells these changes were prevented by amiloride (10^{-5} M), suggesting that the gain of sodium after vasopressin occurs across the mucosal membrane. This hypothesis was confirmed in experiments in which it was found that, in hemibladders mounted in chambers and bathed on their mucosal surface by sodium Ringer's with ^{24}Na, the gains of chemical sodium and ^{24}Na after vasopressin were equivalent.

Neurohypophyseal hormones play an essential role in the maintenance of water balance in many animals. In mammals their effect is principally to promote the passive reabsorption of water from the dilute fluid reaching the distal portions of the nephron. In some amphibia they also affect the water permeability of epithelial membranes such as skin and urinary bladder. This has enabled careful studies to be made of their effects employing the techniques pioneered by Ussing and his collaborators (Ussing, 1960).

These hormones, of which vasopressin is an example, promote not only water but also sodium movement across epithelial membranes such as toad urinary bladder (Leaf & Dempsey, 1960) and frog skin (Ussing & Zerahn, 1951). It has been established that transepithelial transport of sodium is an active process, but the mechanism by which sodium transport is stimulated after exposure to vasopressin has been the cause of some controversy.

Such stimulation might result either from an increased availability of sodium to the active transport site or, alternatively, from a direct stimulatory effect of the hormone on the transport mechanism itself. Frazier, Dempsey, and Leaf (1962) have emphasized that if the stimulatory effects of vasopressin on sodium transport were mediated through an increase in the entry

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of sodium to the cell across the apical or mucosal membrane, then cellular sodium should increase. Conversely, if vasopressin were to act directly on the sodium transport mechanism in the basal-lateral cell membranes, the cellular sodium should decrease.

Attempts to determine the effect of vasopressin on cellular sodium have, however, provided conflicting results. Frazier *et al.* (1962) used isotopic sodium in the medium bathing the mucosal surface of the toad urinary bladder to measure the intracellular sodium "pool" and the influence on it of vasopressin. They demonstrated an increase in labeled tissue sodium after the hormone was added to the serosal medium. In contrast, Janáček and Rybová (1967) performed chemical analyses on frog bladders and obtained results which suggested that oxytocin caused cellular sodium to decrease. Examination of the tissue content of sodium after toad bladders had been exposed to amphotericin B and vasopressin led Finn (1968) to suggest that the decrease in cell sodium produced by the vasopressin under these highly unphysiological circumstances supported the hypothesis that this hormone acted directly on active sodium extrusion from the cell rather than on mucosal permeability alone. Results from such experiments are affected by inclusion of the underlying cells and connective tissue of the submucosa and serosa in the analysis of the composition of the epithelial cells. Furthermore, it has not been possible in experiments with isotopes to distinguish with certainty between isotope within the cells and isotope already transported through the cells but still within the tissue.

More indirect approaches have been employed to determine on which of the two cellular membranes, mucosal or serosal (apical or basal-lateral, respectively), vasopressin exerts its effect. Civan and Frazier (1968) measured the electrical resistances of the membranes and found that the decrease in resistance after vasopressin occurred at the mucosal membrane. Civan, Kedem, and Leaf (1966) found that under conditions of zero net sodium transport, achieved by reducing the electrochemical gradients for chloride ions across the tissue to zero, vasopressin did not regularly increase the transepithelial electrical potential although electrical resistance fell as usual, thereby suggesting that vasopressin acted only on permeability barriers to sodium, and not on the energy-requiring active transport process.

We have recently reported, in preliminary communications (Macknight, Leaf & Civan, 1970*a*, *b*), the effects of vasopressin on the composition of isolated sheets of epithelial cells. The present experiments extend these observations and confirm that, after exposure to vasopressin, cellular sodium, chloride and water increase. Furthermore, it has proved possible to demonstrate that this increase is the result of entry of sodium into the cells from

the mucosal medium alone. It is concluded, therefore, that vasopressin acts to increase sodium entry into the cells from the mucosal medium, thereby making more sodium available to the mechanism concerned in extrusion of sodium from the cells, rather than by directly stimulating the sodium pump itself.

Materials and Methods

The methods employed in these studies are identical with those described in the preceding paper (Macknight, DiBona, Leaf & Civan, 1971) except for some modifications in experiments in which isotopic sodium was used. In these experiments, paired hemibladders were mounted in chambers and both surfaces were bathed by sodium Ringer's. Once short-circuit current (SCC) had become stable, the chambers were drained. The serosal chambers were then filled with sodium Ringer's containing inulin-^{3}H, and the mucosal chambers were filled with sodium Ringer's, containing inulin-^{14}C and ^{24}Na (initial activity 1 mC in 30 ml medium). Hemibladders were exposed for 60 min to these solutions; vasopressin was then added (100 mU/ml) to the serosal medium bathing one hemibladder, and both hemibladders were removed, blotted and scraped 20 min later. Epithelial cells scraped from the hemibladders were transferred to tared plastic tubes which were then reweighed to obtain the wet weight of tissue, and immediately transferred to a Nuclear Chicago Gamma Counter. Samples of both mucosal and serosal medium, collected at 20-min intervals while ^{24}Na was present, were counted at the same time. The plastic tubes were then washed several times with distilled water, the tissue and washings being transferred to borosilicate glass tubes. A glass rod was used to displace any tissue fragments from the walls of the plastic tube, thereby ensuring that all tissue was transferred to the glass tubes. After the tissue and washings from the plastic tubes had been transferred to their respective glass tube, the plastic tubes were washed once more and the washings transferred to a control borosilicate glass tube which was carried through each experiment. Analysis of additional extracts from the plastic tube confirmed that all ions had been removed by the washing.

The glass tubes were placed in a hot air oven at 105 °C and the fluid evaporated to dryness, a process which took several days. They were then cooled at room temperature for 40 min and reweighed to obtain the dry weight of the tissue. Nitric acid, 0.1 M, was added to the dried residue, and the ions and inulin were extracted from the tissue. Tissue extracts and samples of mucosal and serosal medium were then analyzed for sodium, potassium, chloride and inulin using the methods previously described (Macknight *et al.*, 1971). Liquid scintillation counting of inulin was performed after the decay of ^{24}Na.

The ^{24}Na used in these experiments was obtained from Cambridge Nuclear Corporation. The nine experiments with vasopressin and ^{24}Na were performed over three successive days, and the mucosal solutions containing inulin-^{14}C and ^{24}Na were reused over this time. The serosal medium was fresh sodium Ringer's with inulin-^{3}H for each experiment. Vasopressin (Pitressin) was obtained from Parke-Davis.

The content of intracellular Na calculated from ^{24}Na was obtained in the following way.

$$\text{I. C. Na content} = \frac{(\text{intracellular counts})\ (\text{Na}_m) \times 10^3}{(\text{medium counts})\ (\text{tissue d.w.})}$$

where I. C. Na content = mEquiv of sodium per kg tissue dry weight from ^{24}Na; Na_m = μEquiv of sodium per ml of mucosal medium; intracellular counts = total tissue counts per minute corrected for ^{24}Na contamination in the inulin spaces of the mucosal and serosal surfaces; medium counts = counts per minute per ml of mucosal medium; and tissue d. w. = tissue dry weight in mg.

Experiments with ^{24}Na added to the mucosal medium were performed under conditions in which the accumulation in the serosal medium of ^{24}Na, which crossed the epithelium, remained so low that the contribution of ^{24}Na taken up from the serosal medium to total uptake of ^{24}Na was negligible (<2%).

Results

We have previously reported (Macknight *et al.*, 1970*a*) that in isolated sheets of epithelial cells, vasopressin (50–100 mU/ml) increased the cellular contents of sodium, chloride and water, without affecting the content of potassium. This resulted in an increase in cellular sodium and chloride concentrations and in a dilution of potassium concentration. The results were similar whether vasopressin was present for only 5 to 10 min or for 60 min. The viability and responsiveness of the pooled epithelial scrapings were demonstrated by a significant increase in the basal rate of oxygen consumption when vasopressin was added, both after 1 and 2 hr of incubation.

Table 1 shows essentially identical effects of vasopressin on the composition of toad bladder epithelial cells scraped from paired hemibladders mounted in chambers. The hemibladders were bathed with isotonic sodium Ringer's on both surfaces, and after 40 min, when the SCC was stable, one hemibladder was exposed to vasopressin, 100 mU/ml, added to the serosal medium. Once the SCC of the experimental hemibladder had risen and plateaued, a process which took about 20 min, both hemibladders were removed, blotted and the epithelial cells scraped off and taken for analysis. The results shown in Table 1 were obtained from nine experiments performed

Table 1. *The effect of vasopressin on the composition of epithelial cells scraped from paired hemibladders mounted in chambers and bathed on both surfaces by sodium Ringer's. (Nine experiments with nine paired hemibladders)*

	Intracellular water (kg/kg dry wt)	Intracellular ion contents (mEquiv/kg dry wt)			Intracellular ion concentrations (mEquiv/kg I.C. H_2O)		
		Na^+	K^+	Cl^-	Na^+	K^+	Cl^-
Control	3.01 ± 0.18	177 ± 14	428 ± 12	216 ± 14	57 ± 4	144 ± 7	72 ± 2
Vasopressin (100 mU/ml)	3.42 ± 0.14	239 ± 15	419 ± 13	271 ± 14	70 ± 3	123 ± 4	79 ± 2
Difference	0.41 ± 0.13	62 ± 17	9 ± 11	55 ± 9	12 ± 3	21 ± 8	8 ± 2
p	<0.02	<0.01	>0.40	<0.001	<0.01	<0.05	<0.005

Percentage increase in SCC after vasopressin was 148 ± 33%.

in April and May, 1970. Table 5, which will be discussed later, shows the results of similar experiments performed in October, 1970. The mean values differ somewhat. This has been frequently observed when the water and ionic composition of epithelial cells from different batches of toads obtained at different times of the year are compared. However, both sets of experiments show the same significant increases in cellular sodium, chloride and water, without change in cellular potassium confirming previous observations with the isolated sheets of epithelial cells. Table 2 combines the results from both these sets of experiments and reinforces this conclusion. The fluid gained by the cells was clearly isotonic, the concentrations of sodium and chloride of 128 mEquiv per liter not differing appreciably from their concentrations of 117 mEquiv per liter in the medium.

Having confirmed that the presence of vasopressin does indeed increase cellular sodium, chloride and water, we performed experiments to determine whether the sodium entered the cells across their mucosal or serosal membranes. Two approaches were taken to this problem, one using epithelial cell scrapings and the other employing paired bladders mounted in chambers.

Once cells are scraped from the bladder they obviously can no longer be bathed on opposite surfaces by different media but must be exposed on both surfaces to the same medium. It seems, however, that amiloride acts specifically at the mucosal surface to inhibit the entry of sodium to the cells and that at a concentration of 10^{-5} M it has no detectable effect on sodium transport when present only in the medium bathing the serosal surface of the toad bladder (Bentley, 1968; Ehrlich & Crabbe, 1968). Experiments were therefore performed in which isolated sheets of epithelial cells were exposed

Table 2. *The effect of vasopressin on the composition of epithelial cells scraped from paired hemibladders mounted in chambers and bathed on both surfaces by sodium Ringer's. Results of experiments shown in Tables 1 and 5 combined*

	Intracellular water (kg/kg dry wt)	Intracellular ion contents (mEquiv/kg dry wt)			Intracellular ion concentrations (mEquiv/kg I. C. H_2O)		
		Na^+	K^+	Cl^-	Na^+	K^+	Cl^-
Control	3.34 ± 0.12	176 ± 8	436 ± 7	207 ± 9	53.2 ± 2.5	133 ± 5	62.9 ± 2.5
Vasopressin (100 mU/ml)	3.68 ± 0.10	221 ± 9	431 ± 8	250 ± 11	61.0 ± 2.9	118 ± 3	68.7 ± 3.2
Difference	0.34 ± 0.08	44 ± 10	5 ± 6	43 ± 7	7.8 ± 2.1	15 ± 4	5.8 ± 1.3
p	<0.001	<0.001	>0.40	<0.001	<0.005	<0.005	<0.001

Percentage increase in SCC after vasopressin for all 18 observations was 140 ± 23 %.

Table 3. *The effect of amiloride on the composition of isolated sheets of epithelial cells. (Three experiments with 12 observations in each group from pooled cells scraped from 72 hemibladders of 36 toads)*

	Intracellular water (kg/kg dry wt)	Intracellular ion contents (mEquiv/kg dry wt)			Intracellular ion concentrations (mEquiv/kg I.C. H_2O)		
		Na^+	K^+	Cl^-	Na^+	K^+	Cl^-
Control	3.22±0.04	167±6	403±10	222±4	52±2	125±3	69±1
Amiloride (10^{-5} M)	3.14±0.07	163±8	387±10	206±8	52±3	123±2	65±2
p	>0.30	>0.60	>0.20	<0.02	>0.99	>0.50	<0.05

to 10^{-5} M amiloride before or after vasopressin was added to the medium. As a preliminary to these experiments, Table 3 shows the effects of amiloride (10^{-5} M) alone, on the composition of isolated epithelial cells. At this concentration there were no detectable changes in intracellular water, sodium or potassium. There was, however, a small but significant decrease in cellular chloride. (Experiments with amiloride, 10^{-4} M, in the mucosal medium bathing hemibladders mounted in chambers have subsequently revealed that this higher concentration does produce a significant loss of both sodium and chloride from the cells.)

Having demonstrated that amiloride, at a concentration of 10^{-5} M, was without appreciable effect on cellular water or sodium, the experiments summarized in Table 4 were performed. Samples of pooled isolated sheets of epithelial cells were incubated under four conditions. One group was incubated for 50 min in oxygenated sodium Ringer's alone and served as a control. A second group, after an initial 30 min in oxygenated sodium Ringer's, was exposed to vasopressin, 100 mU/ml, for 20 min. A third group was treated similarly to the second but after 10 min with vasopressin, amiloride (10^{-5} M) was added for a further 10-min incubation. In the final group, the procedure was reversed; amiloride (10^{-5} M) was added after 30 min and vasopressin (100 mU/ml) 10 min later. Previous work had shown that the presence of vasopressin (100 mU/ml) for 5 to 10 min was sufficient to produce highly significant changes in the composition of the isolated sheets of epithelial cells (Macknight *et al.*, 1970*a*). The 10-min period with vasopressin should, therefore, have been adequate to produce measurable cellular changes in the present experiments. As Table 4 shows, amiloride (10^{-5} M) was able to prevent or abolish all the expected changes in com-

Table 4. *The effects of vasopressin and amiloride on the composition of isolated sheets of epithelial cells. (Four separate experiments with 18 observations in each group from pooled cells scraped from 96 hemibladders of 48 toads)*

	Intracellular water (kg/kg dry wt)	Intracellular ion contents (mEquiv/kg dry wt)			Intracellular ion concentrations (mEquiv/kg I.C. H_2O)		
		Na^+	K^+	Cl^-	Na^+	K^+	Cl^-
Control	3.47 ± 0.09	199 ± 10	379 ± 15	251 ± 6	58 ± 3	109 ± 4	72 ± 1
Vasopressin (100 mU/ml)	$3.90 \pm 0.09^{a,b}$	$232 \pm 12^{a,b}$	372 ± 11	$276 \pm 10^{a,b}$	61 ± 4	96 ± 2^{a}	71 ± 2
Amiloride (10^{-5} M) then vasopressin (100 mU/ml)	3.67 ± 0.03	189 ± 11	367 ± 12	242 ± 4	52 ± 3	100 ± 3	66 ± 1
Vasopressin (100 mU/ml) then amiloride (10^{-5} M)	3.57 ± 0.08	186 ± 11	371 ± 14	229 ± 5^{a}	53 ± 4	104 ± 3	64 ± 1

[a] Value significantly different from control ($p < 0.05$).
[b] Value significantly different from tissue exposed to amiloride ($p < 0.05$).

position of the epithelial scrapings which were elicited by exposure to vasopressin alone.

Since available evidence indicates that at this concentration amiloride is effective in inhibiting transepithelial transport of sodium only when it is present in the mucosal medium, the results suggest that the vasopressin-mediated increase in cellular sodium is the result of increased entry of sodium across the mucosal cellular membrane.

Two additional experimental approaches were employed using bladders mounted in chambers in an effort to confirm this suggestion.

A series of experiments was performed in which the mucosal surfaces of paired hemibladders were bathed by sodium Ringer's containing inulin-^{14}C while the serosal surfaces were exposed to choline-chloride Ringer's with inulin-^{3}H. Before these solutions were added to the chambers, the serosal medium was changed from sodium Ringer's to choline-chloride Ringer's and 25 to 40 min allowed for the SCC to become stable and for sodium to be washed from the extracellular space and diffuse from the cells. The final concentration of sodium in the choline-chloride Ringer's with inulin-^{3}H at the end of incubation was never more than 2 mEquiv per liter.

Once the bladders were exposed to media containing labeled inulin, about 40 min was allowed for the inulin to become distributed within the extra-

cellular space and vasopressin (100 mU/ml) was then added to one serosal chamber. Once the SCC had risen and plateaued (20 to 25 min), both hemibladders were removed, blotted and the epithelial cells scraped off for analysis. The changes in water, sodium and chloride were slight and, although all were increased after vasopressin, only the gain of sodium by the cells proved to be significant ($p < 0.02$). With no sodium in the serosal medium, sodium moves "downhill" from cell interior to serosal medium. This may account for the smaller gain in cellular sodium content in these experiments than in those performed with the usual concentrations of sodium in the serosal bathing medium. The increase in cellular sodium content does, however, add support to the hypothesis that the increase in sodium content produced by vasopressin is caused by increased entry of sodium into the cells from the mucosal medium.

As an alternative approach to this problem, nine experiments were performed in which bladders were bathed on both their mucosal and serosal surfaces by sodium Ringer's. The serosal medium contained inulin-^{3}H, the mucosal medium inulin-^{14}C and, in addition, ^{24}Na. Sixty min was allowed for the distribution of isotopic sodium in the cells to reach a steady state. That a steady state is achieved in this time is shown by the constancy of mucosal to serosal flux of ^{24}Na over two successive 20-min periods. It has also been found that, under these conditions, with the labeling of one hemibladder from the mucosal medium and the paired hemibladder from the serosal medium, all the chemical sodium in the cell is labeled in 60 min. In addition, the percentage of chemical sodium labeled in the cell from the mucosal medium after 120 min is the same as that labeled after 60 min.

Vasopressin, 100 mU/ml, was then added to the serosal medium bathing one hemibladder while the other hemibladder served as a control. After a further 20 min, when the SCC had risen and plateaued, the hemibladders were removed, blotted and the epithelial cells scraped off for analysis. The results of the experiment are shown in Table 5. As predicted, cells gained water, sodium and chloride with no change in potassium after exposure to the hormone. The intracellular content of sodium in the control cells, as determined with ^{24}Na, was 34 ± 3 mEquiv per kg dry weight (representing $19.3 \pm 1.7\%$ of the chemical sodium content). This increased to 65 ± 6 mEquiv per kg dry weight ($32.5 \pm 3.2\%$ of the chemical sodium content) after vasopressin. The difference of 31 ± 6 mEquiv per kg dry weight was highly significant ($p < 0.001$) and not detectably different from the increase in total intracellular sodium of 26 ± 9 mEquiv per kg dry weight ($p > 0.60$). These results demonstrate that all the sodium gained by the cells after exposure to vasopressin came from the mucosal bathing medium.

Table 5. *The effect of vasopressin on the composition of epithelial cells scraped from paired hemibladders mounted in chambers and bathed on both surfaces by sodium Ringer's. The mucosal medium contained* ^{24}*Na. (Nine experiments with 18 observations in each group from nine hemibladders from nine toads)*

	Intracellular water (kg/kg dry wt)	Intracellular ion contents (mEquiv/kg dry wt)				Intracellular ion concentrations (mEquiv/kg I. C. H_2O)		
		Na	^{24}Na	K^+	Cl^-	Na^+	K^+	Cl^-
Control	3.66±0.08	176±9	34±3	444±7	198±9	47.6±1.5	122±3	54.2±2.
Vasopressin (100 mU/ml)	3.93±0.10	202±6	65±6	443±8	229±16	51.9±1.7	113±3	58.0±3.
Difference	0.27±0.10	26±9	31±6	1±4	31±9	4.3±2.1	9±2.9	3.8±1.
p	<0.025	<0.02	<0.001	>0.80	<0.02	>0.05	<0.02	<0.05

Difference between increase in Na and ^{24}Na after vasopressin was 5±10, $p>0.60$. Percentage increase in SCC after vasopressin was 132±33%.

Discussion

Neurohypophyseal hormones, when added to medium bathing the serosal surface of the urinary bladder of the toad, promote flow of water from the mucosal to the serosal medium when an osmotic gradient exists, increase permeability to urea, and stimulate sodium transport. Present evidence suggests that the action of the hormone is mediated by 3′,5′-cyclic AMP (Orloff & Handler, 1962). The recent demonstration by Hynie and Sharp (1971) of an increase in activity of adenyl cyclase, which catalyzes the conversion of ATP to 3′,5′-cyclic AMP, in preparations from epithelial cells of toad bladders exposed to vasopressin, supports this view.

The present results provide the most direct evidence available in favor of the thesis that, however the effect of vasopressin is mediated within the cells, the major action of this hormone with respect to its effect on sodium transport is to increase entry of that ion into the cells from the mucosal medium. An increase in sodium, of mucosal origin within the epithelial cells, after the addition of vasopressin to medium bathing the serosal surface has been demonstrated. Such a finding is incompatible with the alternative hypothesis that the increase in sodium transport is a direct result of stimulation of the active extrusion of sodium from the cells. Although a minor effect of vasopressin directly on the active extrusion of sodium is not excluded by the present findings, it cannot constitute a major effect nor is it a necessary action of the hormone to account for these results.

It is suggested that intracellular chloride increases to preserve electroneutrality and that water enters the cells in response to the gains in sodium and chloride. This conclusion is supported by the fact that the cells gain an essentially isotonic extracellular fluid. The gain of water leads to a decrease in cellular potassium concentration although the potassium content is unaffected.

The conclusion that vasopressin acts to increase the entry of sodium across the mucosal membrane of the epithelial cells is in agreement with the evidence from electrophysiological studies of the epithelial cells after vasopressin (Civan & Frazier, 1968; Civan, 1970) and with the morphological studies showing that the effect of vasopressin on water flow occurs at the mucosal membrane (Peachey & Rasmussen, 1961; Carasso, Favard & Valérien, 1962; DiBona, Civan & Leaf, 1969).

How the increase in cell sodium induced by vasopressin promotes the active extrusion of sodium from the cells across the basal-lateral boundaries remains to be determined. The limitations of the experimental techniques and the lack of knowledge regarding probable compartmentation of sodium within the epithelial cells prevents an adequate assessment of the problem. Although vasopressin produced a significant increase in cell content of sodium under all circumstances tested, the change in average concentration of sodium within cells did not always show a statistically significant increase. However, concentration is already a derived figure into which are propagated the errors inherent in the measurements of both total sodium content and water content. Even if the increase in the calculated concentration of sodium were significant in all instances, it would not be useful at present to relate changes in the average sodium concentration to changes in sodium transport since the concentration of sodium within the compartment relevant to the active transport of sodium remains unknown.

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