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Time Course of Vasopressin-Induced Formation of Microvilli in Granular Cells of Toad Urinary Bladder

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Summary. Vasopressin-induced transformation of ridges to microvilli on the surface of granular cells of toad urinary bladder occurs in conjunction with induced alterations in the water permeability of the luminal membrane. This study was designed to establish the relationship between the time course for induction of microvilli and the time course for induction of increased water permeability after vasopressin stimulation. Hemibladders were examined at 2.5, 5, 10, 20 and 30 min following exposure to 20 mU/ml of vasopressin and at 5, 10, 20, 30, 40, 50 and 60 min after washout of vasopressin. Within 2.5 min, vasopressin initiated complete transformation of ridges to microvilli on approximately 13% of the granular cells, while osmotic water flow (Jv) was 0.31 ± 0.10 $\mu l \cdot min^{-1} \cdot cm^{-2}$. Five minutes following vasopressin stimulation, microvilli were present on approximately 30% of granular cells and Jv was 2.27 ± 0.13 $\mu l \cdot min^{-1} \cdot cm^{-2}$. At 10 min Jv was maximum at 4.03 ± 0.15 $\mu l \cdot min^{-1} \cdot cm^{-2}$ and 50% of the granular cells were covered with microvilli. This percentage increased to 70% at 20 min and was maintained at 30 min, although Jv decreased to 3.9 ± 0.35 $\mu l \cdot min^{-1} \cdot cm^{-2}$ at 30 min. Five minutes following vasopressin washout, ridges interspersed with microvilli reappeared as Jv fell to 1.10 ± 0.30 $\mu l \cdot min^{-1} \cdot cm^{-2}$. At 10 min after vasopressin washout, Jv approached basal levels, but the reversal of microvilli to ridges remained incomplete. At 60 min after vasopressin washout, the granular cells had regained their original ridgelike surface structures. Thus, these studies establish a temporal relationship between the induction and reversibility of vasopressin-induced microvillous formation and alterations in the osmotic water permeability of the apical plasmalemma.

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Scanning electron microscopy has been used to demonstrate that stimulation of the toad urinary bladder with vasopressin is accompanied by a striking alteration in the surface morphology of the apical membrane of granular cells (Davis, Goodman, Martin, Matthews & Rasmussen, 1974; Spinelli, Grosso & DeSousa, 1974, 1975; Mills & Malick, 1978; Dratwa, LeFurgey & Tisher, 1979*a*). The alteration involves the transformation of branching ridgelike structures normally occurring on the apical surface of granular cells in the absence of vasopressin, to individual microvilli (Figs. 1*a* and 1*b*). This transformation occurs in both the presence and the absence of a transepithelial osmotic gradient and, hence, does not require osmotic water flow (Jv) for its expression (Davis et al., 1974; Dratwa et al., 1979*a*). The findings suggest that the formation of individual microvilli occurs in association with changes in the water permeability of the luminal membrane after vasopressin exposure. Furthermore, the response to vasopressin is mimicked with cyclic adenosine monophosphate (cAMP), again, in both the presence and the absence of a transepithelial osmotic gradient (Dratwa et al., 1979*a*).

The current study was designed to establish the relationship, if any, between the time course for formation of microvilli and that for development of increased osmotic water permeability following vasopressin stimulation, and to evaluate the reversibility of both events following vasopressin washout. It was felt quite likely that these events may be related functionally, since both the morphological and the physiological responses appear to involve alteration of the apical membrane of the granular cell.

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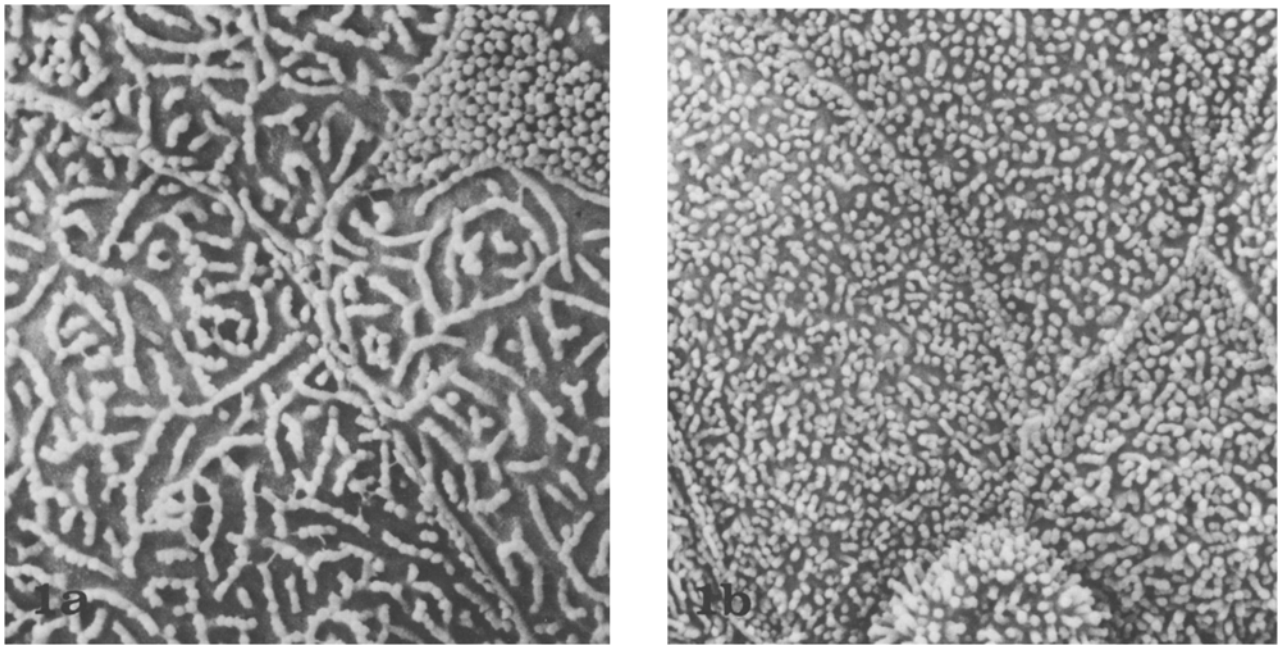

Fig. 1. Scanning electron micrographs of toad urinary bladder. (*a*) Control hemibladder shows granular cells with characteristic ridges on the mucosal surface. A mitochondria-rich cell is shown at the upper right. (×10,000). (*b*) Experimental hemibladder exposed to 20 mU/ml of vasopressin for 30 min shows granular cells with individual microvilli. A goblet cell is located at the lower center. (×10,000)

Material and Methods

The toad *Bufo marinus* was used in all experiments. Paired urinary hemibladders were excised from doubly pithed male toads and immediately placed in aerated amphibian Ringer's solution with the following composition in mmoles/liter: sodium chloride, 111; potassium chloride, 3.4; sodium bicarbonate, 4.0; calcium chloride, 2.7; and dextrose, 5.0. The pH of the solution in equilibrium with room air was 7.8 to 8.0 and the osmolality was 220 to 230 mOsm/kg H_2O.

For most experiments each hemibladder was divided into two portions as previously described (Dratwa et al., 1979*a*). One quarter-bladder was cut into small fragments, 5 mm × 5 mm in size, which were immersed in Petri dishes containing aerated isotonic amphibian Ringer's solution as described above. The remaining quarter-bladder was mounted as a sac on the end of a glass cannula with the serosal surface facing outward (Bentley, 1958). Identical sac and tissue pieces were prepared from the paired hemibladder to serve as controls. The sacs were initially filled with approximately 2 ml of amphibian Ringer's solution diluted 1:4 with distilled water (osmolality: 45 mOsm/kg H_2O) and were immersed in a bath of 18 ml of aerated Ringer's solution (osmolality: 220 to 230 mOsm/kg H_2O).

During the 90-min preincubation of sacs and tissue pieces at room temperature (22 to 24°C), the transepithelial potential difference (PD) across each sac was measured with a Keithley electrometer (model 610 B, Keithley Instruments, Inc., Cleveland, Ohio) using calomel half-cells and agar-KCl bridges as previously described (Croker & Tisher, 1971). The short-circuit current (SCC) was also measured by employing a modification of the method of Ussing and Zerahn (1951). All sacs with a PD of less than 20 mV and the corresponding tissue pieces were discarded.

Physiologic Experiments

To establish the time course for induction of *Jv*, sacs from six hemibladders were incubated in the presence of vasopressin and the osmotic gradient of 180 mOsm/kg H_2O established during the equilibration period. Sacs were transferred into a fresh bath of isosmotic amphibian Ringer's solution containing vasopressin in a concentration of 20 mU/ml (Pitressin, Parke, Davis and Company, Detroit, Mich.). *Jv* was determined gravimetrically (Bentley, 1958) during five intervals: 0 to 2.5, 4 to 6, 9 to 11, 19 to 21, and 29 to 31 min after vasopressin stimulation.

To establish the time course for reversibility of osmotic water permeability, sacs prepared from six additional hemibladders exposed to 20 mU/ml of vasopressin and an osmotic gradient of 180 mOsm/kg H_2O for 30 min were transferred to fresh isosmotic amphibian Ringer's solution and weighed at 4 to 6, 9 to 11, 19 to 21, 29 to 31, 39 to 41, 49 to 51, and 59 to 61 min following vasopressin washout. Paired control sacs were exposed to 20 mU/ml vasopressin for a total of 90 min and weighed at the appropriate 5- or 10-min intervals during the final 60 min of vasopressin exposure. For consistency between control and experimental sacs, one transfer of the control sacs to fresh isosmotic Ringer's solution containing vasopressin was made following the initial 30 min of exposure to vasopressin.

Morphologic Experiments

To establish the pattern for induction of microvillous formation, tissue pieces from the same hemibladders were also exposed to vasopressin. Following a 90-min preincubation in aerated isosmotic amphibian Ringer's solution, tissue fragments from the six hemibladders used in the *Jv* induction experiments were exposed to 20 mU/ml of vasopressin for 2.5, 5, 10, 20 and 30 min before fixation; control tissues were not exposed to vasopressin. To determine the reversibility of microvillous formation, additional tissue fragments from the six hemibladders used in the *Jv* reversibility studies were exposed to vasopressin for 30 min and subsequently fixed at 5, 10, 20, 30, 40, 50 and 60 min after vasopressin washout; controls included tissues not exposed to vasopressin or exposed to vasopressin for 30 min.

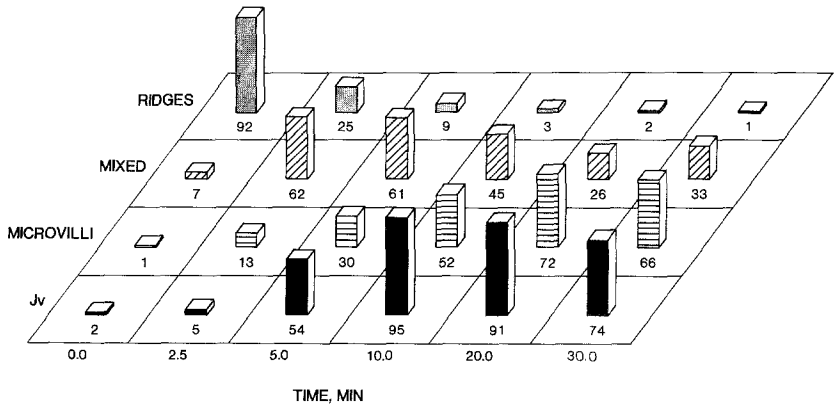

Fig. 2. Time course of the morphological and hydroosmotic response to vasopressin. Vasopressin at a concentration of 20 mU/ml was added to the serosal bath at time 0. Water flow measured gravimetrically in six hemibladders is expressed as the percentage of the maximum *Jv* achieved during the 30-min exposure to vasopressin. Mean percentages of cells with microvilli, ridges, or mixed microvilli/ridges were determined from micrographs of tissue pieces obtained from the same six hemibladders fixed after 2.5, 5, 10, 20 and 30 min of vasopressin exposure. At least 450 cells were counted for each time interval

Scanning Electron Microscopy

Tissue pieces and sacs were fixed for morphological examination by immersion for 30 min in 1.3% glutaraldehyde buffered with 0.05 M sodium cacodylate (pH; 7.4; osmolality, 220 to 230 mOsm/kg H_2O), the osmolality of which matched that of the final bathing solution. After fixation all tissue was rinsed in sodium cacodylate buffer of matching osmolality and stored at 4 °C in the same buffer before dehydration and critical point drying as previously described (Allen & Tisher, 1976). The dried specimens were mounted on aluminium stubs with Dag 154 (graphite in isopropyl alcohol, Ted Pella, Inc., Tustin, Calif.) before coating with gold-palladium to a thickness of approximately 100 Å in a sputter coater (Model E5100, Polaron Equipment, Ltd., Lexington, Pa.). All specimens were examined and photographed with an ETEC "Autoscan" scanning electron microscope (ETEC Corp., Hayward, Calif.) operating at 10 kV. All sacs and tissue pieces were coded so that microscopy and evaluation of micrographs were performed without knowledge of the experimental condition. Five to 10 separate areas, each containing 20 to 25 cells, were selected at random from each specimen for photographic documentation and quantitative cell counts. However, only morphological data derived from tissue pieces were used for statistical analyses.

Statistics

Student's *t*-test was applied to evaluate the differences between the means of paired or, when appropriate, unpaired data (Snedecor & Cochran, 1967) in assessing the vasopressin-induced changes in *Jv*. The significance of differences between percentages of cells with or without microvilli at the time intervals described was examined using the Wilcoxon Rank Sum test and regression analyses (Snedecor & Cochran, 1967). The degree of consistency within any given specimen was determined by the chi square test for homogeneity (Snedecor & Cochran, 1967).

Results

Induction Experiments

The relationship between microvillous formation and *Jv* with time of vasopressin exposure is depicted in Fig. 2 and statistically evaluated in Table 1. At 2.5 min after hormone exposure, approximately 13% of all granular cells were covered with microvilli, while *Jv* was 0.31 ± 0.10 µl·min^{-1}·cm^{-2}. At 5 min approximately 30% of the granular cells were covered with microvilli and *Jv* had increased to 2.27 ± 0.13 µl·min^{-1}·cm^{-2}. At 10 and 20 min *Jv* reached maximum values while transformation was maximum at 20 min. However, during the final 10 min of hormonal stimulation, *Jv* decreased markedly, while the number of cells covered by microvilli did not change significantly. Thus, a similar time course was observed for the induction of osmotic water permeability of the apical membrane and transformation of ridges of individual microvilli.

The morphological process of transformation from ridges to microvilli is depicted in micrographs of tissue pieces fixed at 2.5 min after vasopressin stimulation (Figs. 3*a–c*). The ridgelike surface resembling that of unstimulated cells persisted on approximately 25% of the granular cells 2.5 min after vasopressin

Table 1. Surface patterns and *Jv* during induction phase

Time (min)	Surface pattern[a,b] (%)			Number of cells[f]	*Jv*[a,c] µl·min^{-1}·cm^{-2}
	Ridges	Mixed	Microvilli		
0	91.7 ± 1.4	7.0 ± 1.4	1.2 ± 0.4	948	0.08 ± 0.03
2.5	25.1 ± 8.0 $p < 0.05$[d]	61.5 ± 5.8 $p < 0.05$[d]	13.2 ± 2.4 $p < 0.05$[d]	967	0.31 ± 0.10 NS[d]
5.0	8.9 ± 3.4 $p < 0.05$[d]	61.0 ± 4.6 NS[d]	29.9 ± 7.1 NS[d]	975	2.27 ± 0.13 $p < 0.001$[d]
10.0	2.8 ± 0.9 NS[d]	45.2 ± 3.8 $p < 0.05$[d]	51.8 ± 4.4 $p < 0.05$[d]	972	4.03 ± 0.15 $p < 0.001$[d]
20.0	1.9 ± 1.3 NS[d]	26.0 ± 2.8 $p < 0.05$[d]	71.9 ± 2.3 $p < 0.05$[d]	845	3.91 ± 0.33 NS[d]
30.0	0.8 ± 0.6 NS[d,e]	32.9 ± 3.4 NS[d] $p < 0.05$[e]	66.0 ± 3.2 NS[d] $p < 0.05$[e]	910	3.19 ± 0.35 NS[d] $p < 0.05$[e]

[a] Mean ± standard error.
[b] Statistical comparison, Wilcoxon Rank Sum test.
[c] Statistical comparison, Student's *t*-test.
[d] Compared to the previous time period.
[e] Compared to the 10-min time period.
[f] Number of cells evaluated with scanning electron microscopy.

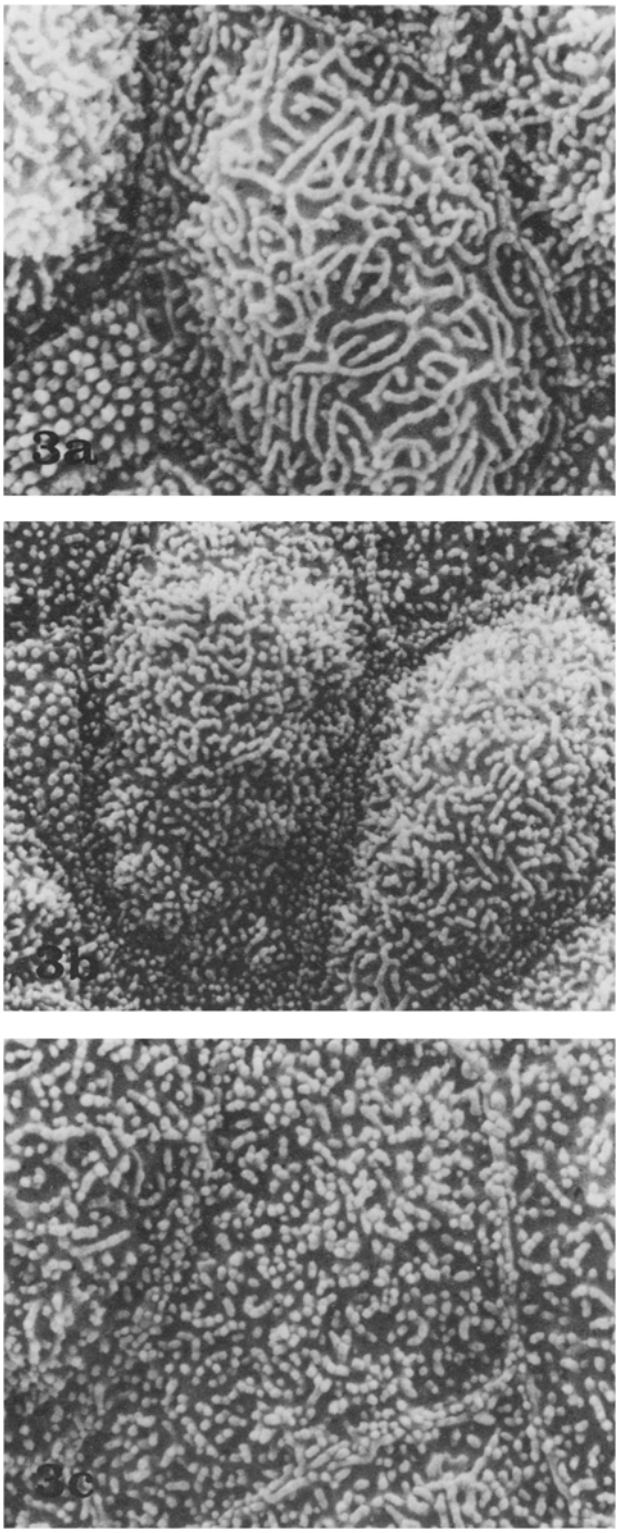

Fig. 3. Scanning electron micrographs of toad urinary bladder exposed to 20 mU/ml of vasopressin for 2.5 min. (*a*) Granular cells which exhibit ridgelike surface structures resembling those of control cells occur 2.5 min following vasopressin exposure. (×10,000). (*b*) In the same hemibladder granular cells also exhibit both microvilli and ridges. (×10,000). (*c*) Other granular cells from the same hemibladder possess only individual microvilli. (×10,000)

Table 2. Surface patterns and *Jv* during reversibility phase

Time (min)	Surface pattern[a,b] (%)			Number of cells[f]	$Jv^{a,c}$ $\mu l \cdot min^{-1} \cdot cm^{-2}$
	Ridges	Mixed	Microvilli		
0	83.3±7.3	10.3± 3.2	6.3±4.7	1703	0.08±0.02
30	1.0± 0.4 $p<0.01$[d]	14.8± 3.1 NS[d]	84.0±3.4 $p<0.01$[d]	1515	1.75±0.22
5W	3.3± 1.2 $p<0.01$[d]	38.4± 7.0 $p<0.01$[d]	58.1±7.8 $p<0.01$[d]	505	1.10±0.30 $p<0.01$[d] NS[e]
10W	8.5± 2.8 $p<0.01$[d]	72.0± 6.5 $p<0.01$[d]	19.3±7.3 $p<0.01$[d]	752	0.35±0.20 NS[d] $p<0.005$[e]
20W	28.6±17.7 $p<0.05$[d]	58.2±14.5 $p<0.01$[d]	13.0±7.6 NS[d]	590	0.52±0.17 $p<0.05$[d]
30W	25.4±11.9 $p<0.01$[d]	55.7±10.5 $p<0.01$[d]	18.7±8.7 $p<0.05$[d]	964	0.44±0.13 $p<0.02$[d]
40W	57.0±12.5 $p<0.05$[d]	38.4± 9.7 $p<0.01$[d]	4.4±3.0 NS[d]	655	0.35±0.08 $p<0.01$[d]
50W	69.8±7.7 NS[d]	28.7± 7.9 $p<0.05$[d]	1.4±0.9 NS[d]	466	0.32±0.11 NS[d]
60W	77.1±3.8 NS[d]	20.1± 3.9 NS[d]	2.7±1.4 NS[d]	551	0.17±0.05 NS[d]

[a] Mean ± standard error.
[b] Statistical comparison, Wilcoxon Rank Sum Test.
[c] Statistical comparison, Student's *t*-test.
[d] Compared to the 0 time period.
[e] Compared to the 30-min time period.
[f] Number of cells evaluated with scanning electron microscopy.

stimulation (Fig. 3*a*). However, the majority of the cells possessed both ridges and microvilli at 2.5 min (Fig. 3*b*). The microvilli first appeared in the more peripheral regions of the apical cell surface, while fragmented ridges persisted more centrally. In cells having undergone complete transformation as early as 2.5 min after stimulation, microvilli covered the entire cell surface (Fig. 3*c*).

The transformation process with time of vasopressin exposure is also summarized in Fig. 2. After 5 min of stimulation cells with ridges represented less than 10% of the total population and decreased to less than 3% during the remaining period of observation. The number of cells in the process of transformation was maximal at 2.5 and 5 min and fell to 26% by 20 min. Cells with microvilli exceeded basal values at 5 min and attained maximal values after 20 min of stimulation when *Jv* was also maximal. During the ensuing 10 min cells with microvilli were predominant.

Reversibility Experiments

Five minutes following vasopressin washout, *Jv* fell to 1.10 ± 0.30 $\mu l \cdot min^{-1} \cdot cm^{-2}$, a value less than half

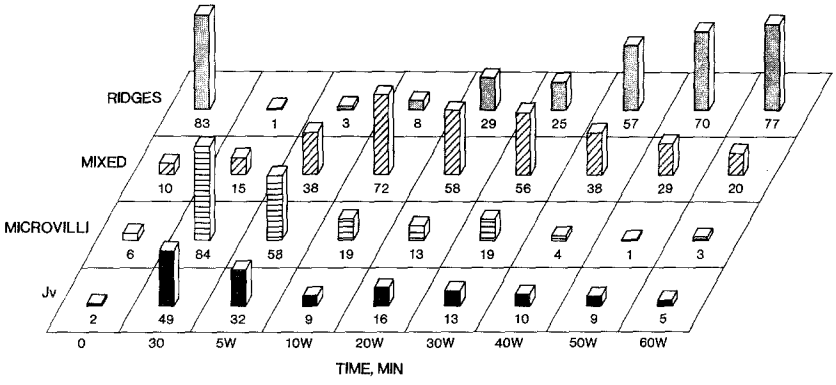

Fig. 4. Time course of the morphological and hydroosmotic response to washout of vasopressin. Water flow measured gravimetrically in six hemibladders at 5- or 10-min intervals following vasopressin removal, is expressed as the percentage of the maximum *Jv* achieved during the 30-min exposure to vasopressin. Mean percentages of cells with microvilli, ridges, or mixed microvilli/ridges were determined from micrographs of tissue pieces obtained from the same six hemibladders fixed after 30 min of vasopressin exposure and 5, 10, 20, 30, 40, 50 or 60 min of washout. At least 450 cells were counted for each time interval

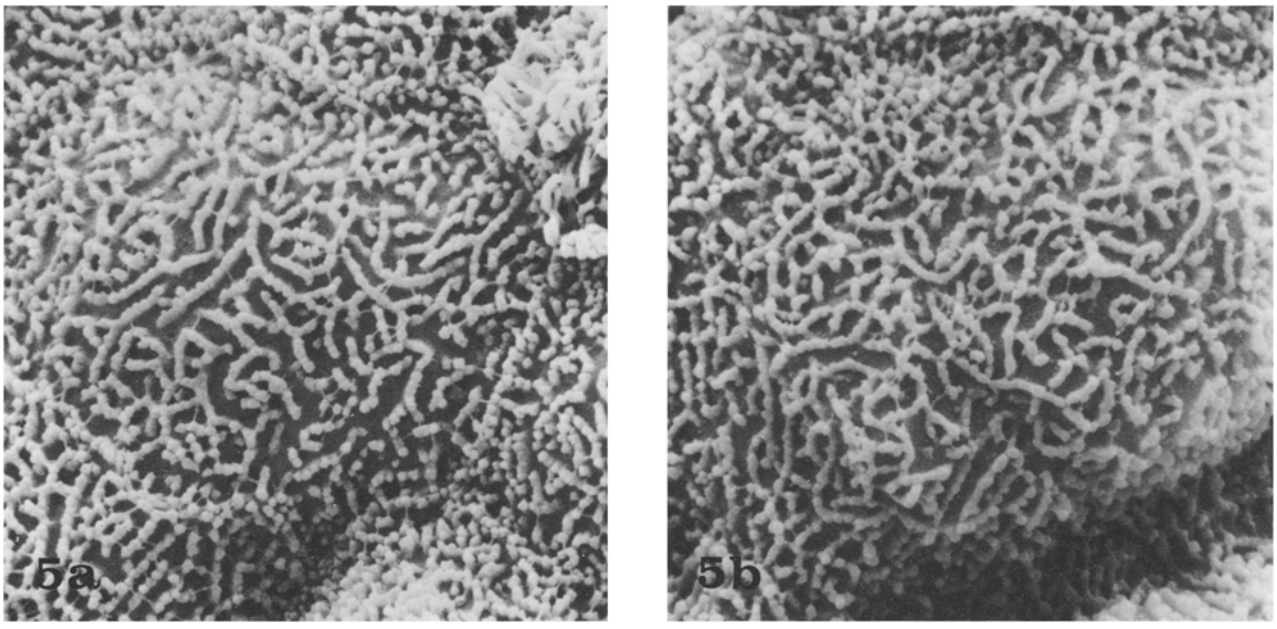

Fig. 5. Scanning electron micrographs of toad urinary bladder exposed to 20 mU/ml of vasopressin for 30 min and rinsed free of vasopressin for 50 min. (*a*) Control hemibladder which has not been exposed to vasopressin shows granular cells with ridges on the mucosal surface. (×10,000). (*b*) Experimental hemibladder exposed to vasopressin for 30 min followed by 60 min of vasopressin washout has granular cells with identical ridgelike structures. (×10,000)

the maximum flow rate, 3.24 ± 0.33 $\mu l \cdot min^{-1} \cdot cm^{-2}$ ($p < 0.001$, $n = 6$), achieved within 10 min after vasopressin stimulation; concurrently 58% of the granular cells were covered with microvilli (Table 2). Ten minutes after washout *Jv* had decreased to 0.35 ± 0.20 $\mu l \cdot min^{-1} \cdot cm^{-2}$, and approximately 20% of the cells remained covered with microvilli. During the remainder of the 60-min observation period, *Jv* continued to approach the basal flow rate of 0.08 ± 0.02 $\mu l \cdot min^{-1} \cdot cm^{-2}$ and cells covered predominately with microvilli fell to less than 5%.

The reversibility phase of the transformation process is summarized graphically in Fig. 4. The reverse process of transformation, that is, from microvilli back to ridges, involved more than one-third of the total cell population at 5 min with 38% of the cells exhibiting some combination of both ridges and microvilli; however, 58% of the cells still exhibited microvilli, while less than 4% had actually regained a ridgelike surface pattern. At 10, 20 and 30 min after washout, 55 to 75% of all granular cells were in a state of transition with both microvilli and ridges or fragmented ridges being present on the apical cell surface. Forty minutes after vasopressin washout, more than 50% of all cells observed possessed ridges, while almost 40% of the cells still remained in the transition state. By 60 min, however, over 75% of the cells exhibited ridges.

The morphological process of transformation from vasopressin-induced microvilli back to ridges is depicted in micrographs of tissue pieces fixed at 60 min after washout of the hormone (Figs. 5*a* and 5*b*). Approximately 85% of cells from control tissue not exposed to vasopressin (Fig. 5*a*) exhibited branching ridges, while 75% of granular cells from tissue exposed to vasopressin for 30 min followed by 60 min of washout had regained the identical surface structure (Fig. 5*b*).

Discussion

The present data suggest that both the induction and reversibility of vasopressin-induced surface alterations of the apical membrane of granular cells in toad urinary bladder generally parallel the changes in osmotic water permeability of the same structure. Microvillous formation and *Jv* were detectable with 2.5 min of vasopressin stimulation, and both phenomena reached maximum values within 10 to 20 min. Five minutes after washout of the hormone, *Jv* was reduced by 50%, and the process of transformation of microvilli back to ridges involved approximately 40% of the total cell population. Ten minutes after washout, *Jv* was approaching basal values and the majority of cells were in transition with their surfaces covered by a combination of microvilli and both intact and fragmented ridges.

It is of interest that the percentage of cells covered by microvilli remained relatively constant at 20 and 30 min after hormonal stimulation, while at 30 min *Jv* had already fallen significantly in comparison to maximum values obtained at 10 min. This so-called "intrinsic" inhibition of *Jv* has been noted by several groups of investigators (Karlin, 1963; Bourguet & Jard, 1964; Edelman, Petersen & Gulyassy, 1964; Schwartz & Walter, 1967; Eggena, Walter & Schwartz, 1968; Eggena, 1972; Kachadorian, Casey & DiScala, 1978; Dratwa, Tisher, Sommer & Croker, 1979*b*) under similar experimental conditions, and as suggested previously in the toad urinary bladder (Kachadorian et al., 1978), may reflect the later development of certain constraints on the cellular diffusion of water that are totally unrelated to the apical membrane of the granular cell. If this is true one would not necessarily expect to see parallel changes in surface structure of the apical membrane as net transepithelial *Jv* decreased. A similar disparity between measurement of *Jv* and intramembranous particle aggregation (IMP) has been reported by Kachadorian and his co-workers (1978).

Further comparison of the induction and reversibility processes reveals differences in the rate at which the majority of cells become covered with microvilli following vasopressin stimulation, and the rapidity with which ridges reappear on the majority of the granular cells following vasopressin washout. At 2.5 and 5 min after hormonal stimulation, approximately 50 to 60% of the cells were already visibly involved in the structural transition from ridges to microvilli. As *Jv* reached a maximum at 10 min, more than 50% of the cells were completely covered with microvilli. In contrast, 5 min following vasopressin washout when 40% of the cells were in a transition phase, less than 5% were actually covered with ridges. This percentage increased to about 10% at 10 min and 30% at 20 to 30 min after vasopressin washout as water flow declined to basal levels. Approximately 40 min were required after hormonal washout before 50% or more of the cells were once again covered with ridges, while most of the remaining cells were still in the state of transition. Thus, the persistence of a large proportion of granular cells in morphological transition occurred at a time when *Jv* was already decreased significantly. It is not clear why the reversal of the morphological response to vasopressin stimulation lags considerably behind the reversal of the osmotic water permeability of the apical membrane. Since the precise mechanism responsible for the cell surface changes is still unknown, any attempt to explain this phenomenon seems premature.

While there does appear to be a temporal relationship between the induction and reversibility of microvillous formation and alterations in the osmotic water permeability of the apical membrane induced by vasopressin, the functional significance of the transformation from ridges to microvilli remains unclear. The transformation may serve to increase the surface area of the granular cells, which could, in turn, facilitate transepithelial water flow. However, it is also possible that the formation of microvilli represents a phenomenon having no direct functional relationship to the permeability of the apical membrane.

Whether any relationship exists between vasopressin- and cAMP-induced microvillous formation and cAMP- and vasopressin-stimulated IMP aggregation in the granular cell is unclear at present. However, it is of interest that the time course for both the induction and reversibility of IMP aggregation (Kachadorian et al., 1978) is similar to that observed for microvilli in the present study. Certain differences do appear to exist, however. For instance, in the induction phase, *Jv*, IMP aggregation and microvillous formation are all submaximal at 2.5 min after vasopressin stimulation (Kachadorian et al., 1978; present study). However, IMP aggregation appears to maximize at 5 min following hormonal stimulation (Dratwa & Tisher, 1979), while, as shown in the present study, microvillous formation and *Jv* require approxi-

mately 10 to 20 min of hormonal stimulation to reach maximum values. Thus, maximization of IMP aggregation appears to precede that of microvillous formation as well as *Jv*. Observations on the time course for the reversibility of IMP aggregation following vasopressin washout are not sufficiently detailed (Kachadorian et al., 1978) to permit extensive comparison with the results of the present study. However, it does appear that the rate of reversibility of IMP aggregation exceeds that for the reappearance of ridges on the surface of the granular cells.

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