Effect of Temperature on Apical Membrane Remodeling in ADH-Stimulated Toad Urinary Bladders (44297)

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Abstract. **Pretreatment and removal of vasopressin (ADH) in toad urinary bladder renal model tissues induces endocytosis at 25°C. The objective of the current study is to determine if apical membrane remodeling, as well as transepithelial water flow, can be affected by lowering the temperature to 15°C. Control toad urinary bladders in the presence of an osmotic gradient at either 25°C or 15°C when visualized by scanning electron microscopy (SEM) show a typical apical membrane surface with no apparent surface differences. ADH-treated tissues following 15-min stimulation at 25°C or 15°C revealed a propagation of apical microvilli on their surface membranes. After 15 min following removal of ADH, bladder tissues at 25°C or 15°C showed surface invaginations involving over 44% and 80% of granular cells, respectively. The rate of water flow in tissues at 15°C remained elevated compared to tissues held at 25°C. This was consistent with the observation that ADH-stimulated tissues following washout at 15°C still had marked apical membrane surface involvement. However, at 30 min and 60 min postwashout, ADH-stimulated tissues at 15°C recovered considerably, with a reduction in the number of shallow apical membrane invaginations involving fewer than 33% and 20% of granular cells respectively. This may indicate that the membrane undergoes continuous remodeling even at cold temperature conditions but with a different half-time. Control bladder tissues subjected to transmission electron microscopy (TEM) reveal a dense cytoplasmic profile with a scattered distribution of secretory granules, rough ER cisternae, mitochondria, and little or no vacuolation. In contrast, ADH-stimulated bladder tissues displayed a vacuolated cytoplasm, expanded rough ER cisternae, and ruffled basolateral membranes. These observations suggest that the apical membrane undergoes considerable reorganization following cessation of hormone action and that lowering the temperature reduces the rate of membrane remodeling and thus may provide a means to monitor the processes of endocytosis and the mechanisms responsible for water channel retrieval.** [P.S.E.B.M. 1998, Vol 218]

Eukaryotic cells respond with membrane remodeling as part of a number of cellular processes. Exo- and endocytosis are two important cellular mechanisms that are involved in the regulation of enhanced transmembrane osmotic water flow and cycling of water channels in renal and toad urinary bladder granular epithelia following stimulation with vasopressin. Vasopressin induces exocytosis with insertion of water channels (1–6) in concert with propagation of numerous microvilli over the apical membranes of the granular cells (7–13). As a result, the apical membrane undergoes enhanced capacitance (14) and conformational changes (15) during enhanced water flow. Following cessation of hormone actions or receptor downregulation, endocytosis restores the apical membrane surface area, and water channels are assimilated into the cytosol (16–24). Little information exists concerning the effect of temperature on exo- and endocytosis at the apical membrane and its corresponding changes in the cytoplasmic ultrastructure during ADH-mediated water flow.

Previously, we reported preliminary findings concerning time-dependent experimental induction of endocytosis

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and apical membrane remodeling at two temperatures (25°C and 15°C) that corresponded with the rate of water flow in ADH-challenged toad urinary bladders (26). Since endocytosis is a highly regulated process and is sensitive to temperature changes (27), we conducted the current series of experiments at 25°C and 15°C to determine the effects of temperature on the morphological changes accompanying ADH action in toad urinary bladders. Many cellular processes, including the fusion of pinocytic vesicles and lysosomes, are temperature sensitive (28, 29) and inhibited selectively at temperatures below 17°C. Poikilothermic animals, like amphibians, must adapt to changes in temperature rapidly during normal life cycles as well as during periods of hibernation. This adaptation must include the ability of the animals to osmoregulate under varied temperatures. Experiments were therefore conducted to analyze the effect of lowering the temperature to 15°C on endocytosis and water flow, as well as the number and size of membrane invaginations seen following stimulation and removal of vasopressin.

Materials and Methods

Animals. Tropical toads, *Bufo marinus,* purchased from Carolina Biological Supply Company (Burlington, NC) were maintained in an aquatic environment irrigated with a fresh supply of tap water, and were fed live crickets biweekly.

Experimental Protocol. Urinary hemibladders were removed surgically from doubly pithed toads, and were set up as sacs at the ends of glass tubes and equilibrated in aerated normal Ringer's solution for 15 min at 25 ± 1°C or 15 ± 1°C in a thermostatically controlled water bath prior to experimental procedures. The composition of the Ringer's solution was as follows (in millimoles per liter): NaCl, 111; KCl, 3.35; CaCl, 2.7; MgCl, 0.5; NaHCO3, 4.0; and glucose, 5.0 with pH adjusted at 8.0. The Ringer's solution was continuously aerated with room air throughout the experimental procedures. Paired hemibladder sacs were used as control and experimental tissues. An osmotic gradient was established between the serosal and mucosal sides using a 1/10 dilution of Ringer's solution of the mucosal cavity. Vasopressin at a concentration of 100 mU/ml was then added to the serosal bathing solution of the experimental bladder sacs for 15 min as in previous studies (21, 22). ADH stimulation for 10 or 15 min at room temperature induces maximum water flow as well as membrane fusion events with a limited membrane downregulation in toad urinary bladders (9, 10, 16, 19, 22, 30), whereas prolonged ADH stimulation causes spontaneous apical membrane remodeling and return to a state similar to reactivation (25). A period of 15 min for ADH stimulation was used for most of our experiments including the current studies.

Following stimulation of bladder sacs with ADH, both control and experimental bladder sacs received two quick serosal buffer rinses at the appropriate temperature for withdrawal of hormone action, and the sacs were then allowed to recover or retrieve for 15 min, 30 min, or 60 min to allow for endocytosis and apical membrane remodeling to occur (22). These experiments were performed at 25°C and 15°C prior to tissue fixation. Before tissue fixation, osmotic water flow was measured gravimetrically (31) at various intervals under the different temperatures. For tissue fixation, the whole hemibladder sac was removed from the end of the glass tube and quickly submerged into 2% glutaraldehyde at the matched temperature and subsequently fixed for 1 hr. Following buffer rinses, postfixation was carried out using 1% osmium tetroxide at room temperature for an additional hour. These tissues were then divided into samples for SEM and TEM preparations.

Tissue Preparations for Electron Microscopy. Tissue processing for SEM and TEM was carried out as described previously (10, 22). For comparative analysis, all SEM pictures were taken at 1500x and at 3000x magnifications with appropriate increases in the printing images. For TEM studies, fixed bladder sacs were buffer rinsed, minced into small pieces, and processed for embedding in epon for ultrathin sectioning (10, 22). Tissue blocks were polymerized overnight in an oven at 60°C. Ultrathin sections made with a diamond knife were collected on bare copper or nickel grids and exposed to uranyl acetate and lead citrate for staining and TEM studies (22). All TEM pictures were made at 31,500x, unless otherwise noted.

Determination of Percent of Surface Involvement. For analysis of the percentage of cells showing surface invaginations, tissues were selected randomly, and scanning electron micrographs were taken at 1500x and 3000x magnifications from areas with clear cellular delineations as described previously (22). As before, the total number of normal and invaginated cells for each treatment were counted separately and averaged to show the results in percentages (Fig. 1).

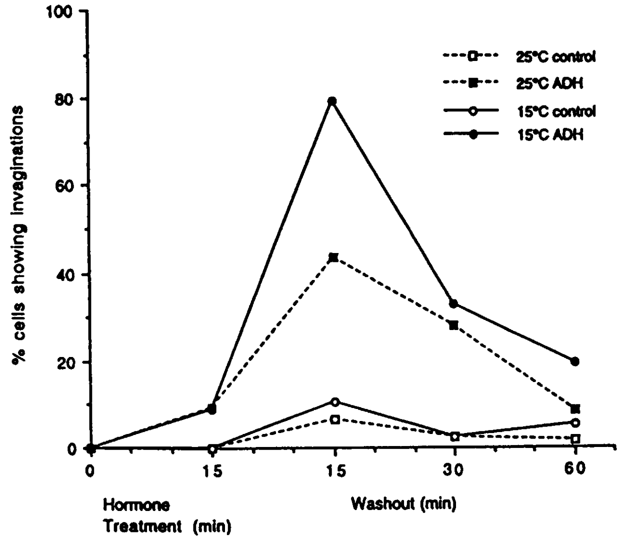

Figure 1. Shows the effect of temperature on percent of invaginations at 25°C and 15°C in the control and ADH-stimulated TUB sacs at 15 min exocytosis and at 15, 30 and 60 min washout periods.

Drugs. Vasopressin 8-Arginine was purchased from Sigma Chemical Co. (St. Louis, MO).

Results

Recently, we reported a time-dependent experimental induction of endocytosis and changes in the rate of transcellular osmotic water flow in toad urinary bladders at 25°C (21, 22, 25). Since endocytosis is a highly regulated cellular process and is reported to be sensitive to temperature fluctuation, a series of experiments was completed to evaluate the sequence of morphological events of endocytosis and apical membrane remodeling that occurs following vasopressin treatment and its removal at 25°C versus 15°C for 15 min, 30 min, or 60 min. The microstructure changes at the apical membrane surface and the cytoplasm, associated with the effects of temperature and hormone, were assessed using the techniques of scanning (SEM) and transmission electron microscopy (TEM). An SEM image from a control urinary hemibladder sac, fixed instantly in glutaraldehyde upon its isolation from a doubly pithed toad is presented in Figure 2a. This procedure captures the apical membrane morphology in nearly its normal state, showing a flat membrane surface configuration containing predominant microridges. This prominent microridge structure was also shown *in vitro* in control bladder tissues set up as sacs for 30 min using Ringer's solution with an imposed osmotic gradient. However, the *in vitro* bladder sacs showed some degree of swelling of granular cells due to the imposed osmotic gradient. Similar to previous reports (21, 22), SEM observations of the control tissues (no hormone) showed surface microstructures as predominantly composed of microridges with no evidence of membrane surface invagination typically indicative of endocytosis (data not shown). Complementary bladder tissue subjected to transmission electron microscopic (TEM) studies displayed the cytoplasmic profiles as composed of dense secretory granules, mitochondria, and microfilaments with basolateral membranes containing slight indentations with intact desmosomes. Little or no apparent difference in cytoplasmic profile was found as was similarly reported in earlier studies (10). Repeating this experimental procedure at 15°C with a 15-min recovery period in control tissues (no hormone) produced no marked discernable morphological differences from 25°C. Figure 2b represents a TEM image of a control tissue retained in Ringer's solution at 15°C following the 15-min recovery period showing a typical cytoplasmic profile. The electron-dense secretory granules (curved arrows) appear scattered within the cytoplasm mingled with mitochondria (arrowheads) rough ER cisternae (short arrows), a golgi body (long arrow), a multivesicular body (b), microtubules, and evenly distributed microfilaments. The slightly indented basolateral membranes with intact desmosomes (d) and nucleus (n) appear normal for the control toad urinary bladder tissues. In contrast, toad urinary bladder sacs stimulated with 100 mU/ml ADH for 15 min at 25°C or 15°C when examined in the SEM showed propagation of numerous microvilli over the apical membranes as presented in Figures 3a and b (arrows) respectively. At 25°C or at 15°C during 15-min exocytosis, ADH-stimulated tissues may undergo some endocytosis as depicted by shallow depressions over the apical membranes (arrows) and separations of the basolateral membranes of the granular cells (Fig. 4a). Analysis of the apical membrane surfaces made from SEM micrographs representing both control ($n = 6$) and ADH-stimulated ($n = 6$) tissues at 25°C and 15°C indicated that no more than 10% of the granular cells showed evidence of surface membrane invaginations in ADH-challenged tissues during the 15-min treatment at 25°C or 15°C, whereas control tissues showed none (Fig. 1). Osmotic water flow was significantly increased in ADH-stimulated tissues as compared to control tissues during this 15-min stimulation period (Table I).

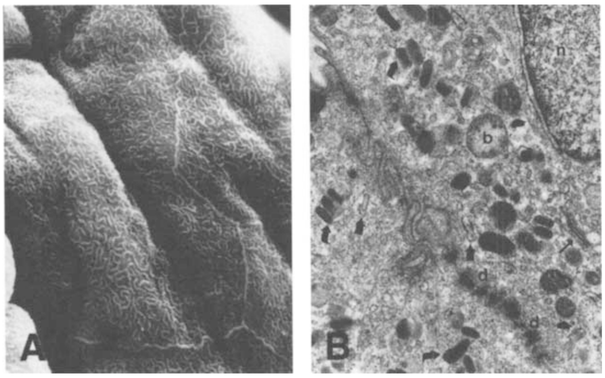

Figure 2. (*a*) SEM of control toad urinary bladder fixed immediately upon surgical removal with no experimental manipulation showing the flat surface image of the apical membrane with predominant distribution of microridges. 3750×. (*b*) TEM of control toad urinary bladder granular epithelial cells retained at 15°C for 15 min, followed by buffer rinses to allow for a 15-min recovery. TEM shows basolateral membranes with intact desmosomes (d), electron dense secretory granules (curved arrows), mitochondria (arrowheads), rough ER cisternae (short arrows), a golgi body (long arrow), nucleus (n), and a multivesicular body (b) within the dense cytoplasmic profile of these cells. 12,500×.

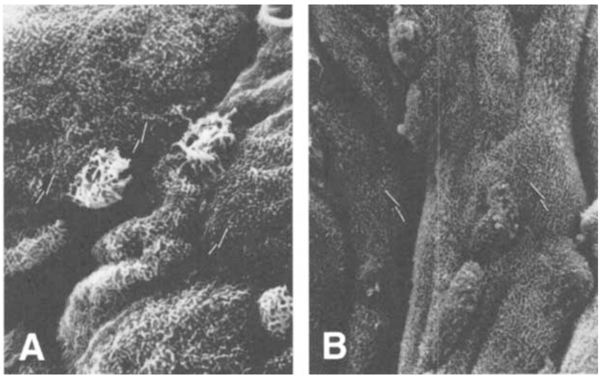

Figure 3. (*a*) SEM of toad urinary bladder stimulated with 100 mU/ml ADH for 15 min at 25°C showing propagation of numerous short microvilli (arrows) over the apical membranes of the granular cells during exocytosis. 3750×. (*b*) SEM of toad urinary bladder stimulated with 100 mU/ml ADH for 15 min at 15°C showing the propagation of numerous microvilli (arrows) over the apical membranes of the granular cells during exocytosis. 3750×.

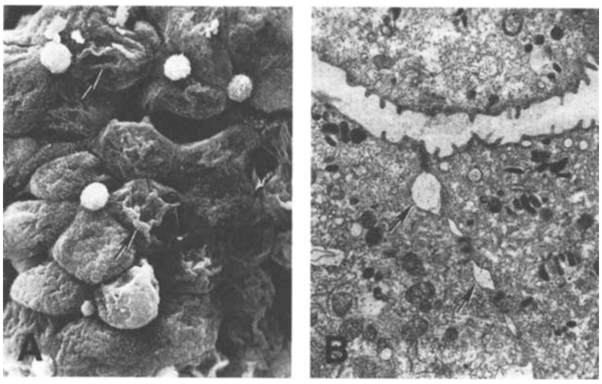

Figure 4. (*a*) SEM of toad urinary bladder stimulated with 100 mU/ml ADH for 15 min at 15°C showing at times the presence of shallow depressions (arrows) over the apical membranes of the granular cells during exocytosis. 3750×. (*b*) TEM of toad urinary bladder tissue at 15°C showing scattered distribution of cellular organelles along with slight separations of the basolateral membranes (arrows). 6250×.

These findings appear to correlate with results from previous studies showing no more than 15% of the granular cells with membrane invaginations at room temperature (25°C). Ultrathin sections of tissues stimulated with ADH for 15 min at 15°C revealed a cytoplasmic composition similar to that reported previously with ADH, including evidence of separations of basolateral membranes (Fig. 4b, arrows).

The above studies of hormone treatment and exocytosis were complemented with studies of endocytosis following washout at the corresponding temperatures and periods of 25°C or 15°C for 15-min, 30-min, and 60-min postwashout periods. Both the control and the ADH-exposed tissues, following incubation for 15 min at 25°C or 15°C, received two quick serosal buffer rinses to remove ADH from the ADH-exposed tissues. These tissues were then allowed to recover for 15 min, 30 min, or 60 min to allow for membrane remodeling and endocytosis of water channels. Figure 5a represents an example of SEM images showing invaginations at the apical surface with a loss of membrane microstructures in ADH-exposed tissues during the 15-min recovery period at 25°C. In some cases, the invaginations involved the entire apical membrane surface with a formation of large cavities as shown in Figure 5b. Ultrathin sections show considerable cellular reorientation producing large inter- and intracellular vacuoles (Fig. 6a, v). The basolateral membranes were not found to be separated at points where desmosomes were present (Fig. 6b, arrows), but intracellular organelles, including the microfilaments, became highly condensed within the cytoplasm (Fig. 10, arrowheads). However, the apical membrane during a 15-min recovery period at 25°C showed predominant microridge structure similar to control tissues before washout (Fig. 6b). Control tissues at 15°C at the 15-min washout period showed some degree of apical membrane invagination (Fig. 7a, arrow) while maintaining the predominant microridge structure. However, in some instances, control tissues showed some degree of surface invagination during a 15-min recovery period at 15°C (Fig. 7b, arrows), indicating that low temperature had some effect on membrane remodeling. In contrast, ADH-exposed urinary bladder sacs at 15°C at the 15-min recovery period showed a dramatic expression of surface membrane effects, with membrane invaginations involving a large number of granular cells (Fig. 7c, Fig. 1). Water flow in these tissues remained considerably elevated compared to other ADH-challenged tissues at 25°C (Table I), indicating that the water channels may not have all been internalized as endosomes during this washout period at the lower temperature. A closer SEM view further amplifies the shallow apical membrane profiles with associated short microvilli and deep caving of the basolateral membranes (Fig. 7d).

Studies of the ADH-stimulated tissues at 15°C at the 15-min recovery period showed that some granular cells underwent considerable reorientation and displacement of the cellular organelles from their normal cellular distribution (Fig. 8a). Most of the secretory granules (arrows) appear to have been displaced from the subapical region deep into the cytoplasm. In some cases, the lower temperature effect on ADH-stimulated tissues at 15°C during the 15-min recovery period caused considerable alterations in the basolateral membranes (Fig. 8b). Additionally, microfilaments are seen to have clustered (arrows) between the compressed basolateral membranes. However, the sheer forces of compressions at the basolateral membranes failed to cause a complete separation of the adjacent cells at points of desmosome attachments (Fig. 7, large arrows). These tissues also had accumulation of a large number of electron dense secretory granules in some granular cells (arrows) as seen in Figure 8c. The control bladder tissues at 15°C during the 15-min washout period showed a different cytoplasmic composition pattern with a scattered distribution of the cellular organelles (Fig. 8d, arrows).

We have also evaluated the apical membrane of the control and ADH-challenged toad urinary bladder tissues at 30 min and 60 min following washout at 25°C and 15°C. Figure 9a illustrates a global SEM image of the control tissues at a 30-min recovery period at 25°C showing a large number of granular (arrows) and goblet cells (small arrows) with little or no apparent sign of apical invaginations. Tissues treated with ADH at 25°C following a 30-min recovery period showed surface invaginations involving a large number of granular cells (Fig. 9b). Longer recovery times of 60 min for control tissues at 25°C or 15°C revealed almost the same apical membrane surface image as that of other control tissues (see Figs. 6b, 9a). ADH-stimulated tissues following a 60-min washout period at 25°C showed almost complete apical membrane remodeling back to prehormone states such that less than 9% of the granular cells showed surface invaginations (Fig. 9c, arrows). In contrast, ADH-stimulated tissues at 15°C during a similar 60-min washout period showed larger membrane surface effects (Fig. 9d, arrows) involving twice the number of granular cells (19%) as that of the ADH-stimualted tissues at 25°C (Fig. 1).

Table I. Effect of Temperature on Osmotic Water Flow Recovery Following ADH-Treatment and Washout

Temperature	Period I	Period II		
	Hormone Treatment	Washout		
	15 min	15 min	30 min	60 min
A. 25°C				
Control	104 ± 13 (12)	78 ± 29 (6)	37 ± 9 (6)	40 ± 9 (6)
ADH	851 ± 190(12)	796 ± 59 (6)	489 ± 92(6)	172 ± 29(6)
B. 15°C				
Control	127 ± 15 (12)	79 ± 28 (6)	71 ± 18(6)	54 ± 14(5)
ADH	611 ± 95 (12)	870 ± 176(6)	*832 ± 56(6)	*448 ± 66(5)

Note. The number of experiments is in parentheses. Water flow as mg/30 min.
Represented as means ± S.E.
Period I represents 15 min following treatment with ADH or without ADH.
Period II represents time periods post-washout of hormone.
* $P < .05$ for differences from 25°C treatment using a Student's t-test.

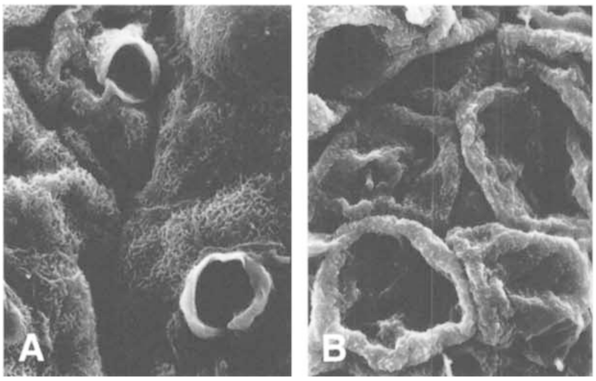

Figure 5. (*a*)SEM of toad urinary bladder stimulated with 100 mU/ml ADH for 15 min at 25°C, buffer rinsed and allowed retrieval for 15 min showing invaginations over the apical membranes of the granular cells indicating possible endocytosis. 3750x. (*b*) SEM of toad urinary bladder stimulated with 100 mU/ml ADH for 15 min at 25°C, buffer rinsed and then allowed to retrieve for 15 min showing large invaginations over the entire apical membranes of the granular cells during endocytosis. 3750x.

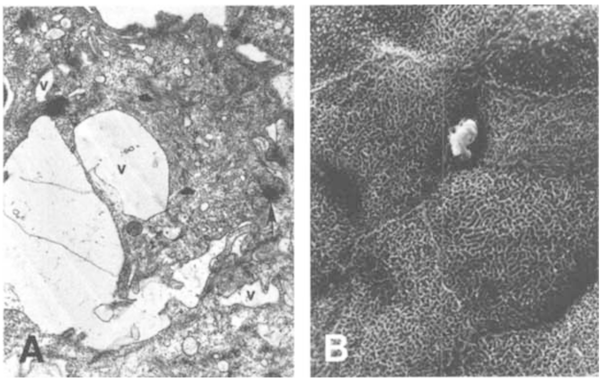

Figure 6. (*a*) TEM of toad urinary bladder stimulated with 100 mU/ml ADH at 25°C, buffer rinsed and then retrieved for 20 min showing the presence of small and large inter- and intracellular vacuoles (v), condensed microfilaments (arrowheads), reorientation of the cells with a displacement of desmosomes (arrows). 6250x. (*b*) SEM of control toad urinary bladder retained in Ringer's solution at 25°C, buffer rinsed and then retrieved for 15 min showing predominant distribution of microridges over the apical membranes with no sign of membrane invagination and endocytosis. 3750x.

An analysis of the percentage of granular cells, both in the control and ADH-stimulated tissues, showing surface invaginations at 25°C and 15°C during 15 min of stimulation and following 15-, 30- and 60-min washout periods has been carried out using SEM images (see Fig. 1). Control tissues retained at 25°C and 15°C for 15 min showed no sign of apical membrane surface depression indicative of endocytosis. The ADH-exposed tissues at the same temperatures for 15 min exocytosis showed nearly 10% of the granular cells with surface invaginations, indicating that even during ADH stimulation, some degree of membrane remodeling occurs that may reflect cycling of water channels during hydro-osmosis. Control tissues at 25°C ($n = 13$) and 15°C ($n = 15$) during a 15-min washout period showed some apical membrane remodeling although involving fewer than 6% and 11% of the cells (Fig. 1). In contrast, ADH-challenged urinary bladder tissues at 25°C ($n = 17$) and 15°C ($n = 15$) during a 15-min recovery period showed a dramatic effect on membrane invagination, with 44% and 80% of granular cells showing membrane surface invaginations at 25°C and 15°C respectively (Fig. 1). Statistical analysis using an ANOVA and Fischer's PLSD showed that there was no significant difference between any of the controls at either 25°C or 15°C during 15-min exocytosis or at 15-, 30-, and 60-min washout periods. In contrast, ADH-stimulated tissues showed significant differences ($P < .05$) in tissues at 25°C and at 15°C during the 15-min recovery period, indicating that cold temperature resulted in an increase or retention in the number of invaginations at the apical plasma membranes of the granular cells. The membrane surface collapse or invaginations appeared to be very shallow at 15°C showing little or no sign of membrane internalization. Therefore, the water channels in the ADH-challenged tissues at 15°C during the 15-min recovery period may not have been internalized into the cytoplasm as endosomes. This may be a reason why we observed a sustained elevated water flow for tissues at 15°C even at 60 min postwashout (Table I). SEM observations of the control tissues at 15°C during 30- or 60-min washout, revealed only 2%–5% of the granular cells showing signs of endocytosis at the apical membrane surface. This was com-

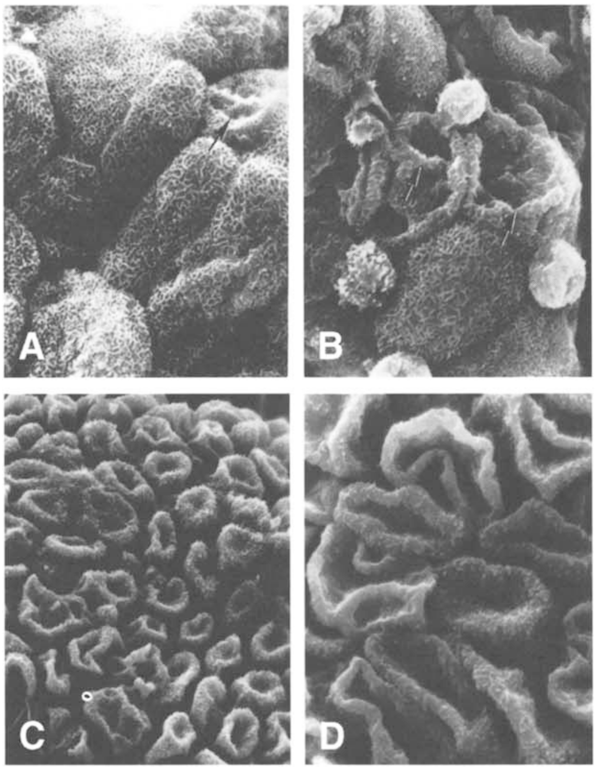

Figure 7. (*a*) SEM of control toad urinary bladder retained in Ringer's solution for 15 min at 15°C, buffer rinsed and then retrieved for 15 min showing slight depressions (arrow) over the apical membranes during endocytosis. 3750×. (*b*) SEM of control toad urinary bladder retained in Ringer's solution for 15 min at 15°C, buffer rinsed and then retrieved for 15 min showing the induction of invaginations over the apical membranes due to cold temperature effect during endocytosis. 3750×. (*c*) SEM of toad urinary bladder stimulated with 100 mU/ml ADH for 15 min at 15°C, buffer rinsed and then retrieved for 15 min showing invaginations and caving of the basolateral membranes of the granular cells. 1875×. (*d*) An enhanced view of Fig. 8a showing the detailed microstructure morphology of the apical membranes of the granular cells with invaginations and dwarf microvilli (arrows). 3750×.

parable to control tissues observed at 25°C during 30- and 60-min similar washout periods (22). In contrast, ADH-challenged tissues at 25°C or at 15°C for the same periods demonstrated considerable membrane surface invaginations involving a number of granular cells. The percentage of granular cells showing the presence of invaginations over the apical membranes at 25°C ($n = 5$) was over 28% (30 min) and 9% (60 min) ($n = 5$) and at 15°C was over 33% (30 min) (n = 7) and 20% (60 min) ($n = 7$) in ADH-stimulated tissues respectively, versus less than 2% (30 min) ($n = 5$) and 6% (60 min) ($n = 8$) in comparable control bladder cells. The results indicate that ADH-stimulated tissues continue to show faster apical membrane remodeling during a 60-min recovery period at 25°C compared to tissues at 15°C (Fig. 1). For this reason, the rate of water flow at 25°C and at the 60-min recovery period was considerably lower than the tissues at 15°C at a similar recovery time (Table I).

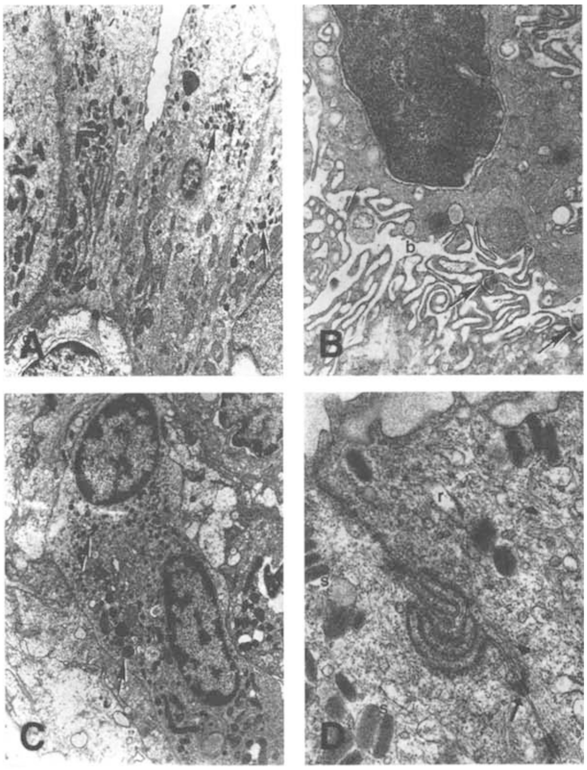

Figure 8. (*a*) TEM of toad urinary bladder tissue stimulated with 100 mU/ml ADH at 15°C for 15 min, buffer rinsed and then allowed to retrieve for 15 min. Micrograph depicts cellular elongation with a caving in the basolateral membranes and a longitudinal distribution of cellular organelles including the rough ER, mitochondria with a displacement of the electron dense secretory granules (arrows) from the sub-apical region to deeper region of the cytoplasm. 6250×. (*b*) TEM of toad urinary bladder stimulated with ADH for 15 min at 15°C, followed by buffer rinses to allow 15-min recovery. Micrograph demonstrates the extensive infoldings of the basolateral membranes (b) with displacements of desmosomes (large arrows) and clustering of microfilaments (arrows) as a result of compression likely caused by ADH treatment at cold temperature of 15°C. 12,500×. (*c*) TEM of toad urinary bladder stimulated with ADH at 15°C during 15-min retrieval period following buffer rinses showing an accumulation of a large number of electron-dense secretory granules (arrows). 6250×. (*d*) TEM of control toad urinary bladder tissue retained at 15°C for 15 min, followed by buffer rinses to allow for a 15-min recovery. Figure shows the cytoplasmic profile of the cells with a scattered distribution of microfilaments (arrowheads), secretory granules (s), rough ER cisternae (r), and basolateral membranes with slight infoldings and intact desmosomes (arrows). 25,000×.

Discussion

The kidney plays a critical role in the reabsorption of water from the urinary side into the systemic circulation, and at times of thirst this is enhanced by antidiuretic hormone (ADH). The process of transcellular enhanced water reabsorption by ADH results from the insertion of water channels into the apical membrane. This ADH effect is seen in many ADH responsive renal tissues, including the toad urinary bladder. Upon serosal stimulation of the toad urinary bladder tissues with ADH, the apical membranes of the granular epithelia become highly water permeable following membrane fusion of water channels during a process of

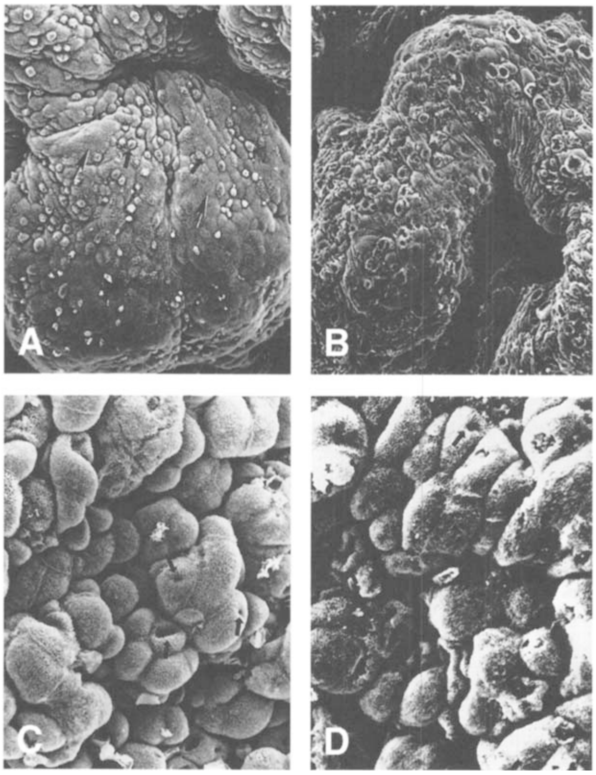

Figure 9. (*a*) SEM of control toad urinary bladder retained in Ringer's solution for 15 min at 25°C, buffer rinsed and then retrieved for 30 min showing the global view with tissue swelling under osmotic gradient and no evidence of apical membrane surface invagination indicative of endocytosis. 500×. (*b*) SEM of toad urinary bladder stimulated with ADH for 15 min at 25°C, buffer rinsed and then retrieved for 30 min showing the global view of the apical membrane surface invaginations involving a large number of granular cells and making the cells porous. 500×. (*c*) SEM of toad urinary bladder stimulated with 100 mU/ml ADH for 15 min at 25°C, buffer rinsed and then retrieved for 60 min showing almost complete restoration of the apical membrane with few cells still showing small invaginations (arrows). 3750×. (*d*) SEM of toad urinary bladder stimulated with 100 mU/ml ADH for 15 min at 15°C, buffer rinsed and retrieved for 60 min showing retention of shallow invaginations (arrows) likely without retrieving the water channels. 3750×.

exocytosis. Following exocytosis and the removal of the hormone, the apical membrane undergoes spontaneous recovery, and water channels are retrieved into the cytoplasm by a process of endocytosis (3, 22). A number of studies have probed the process of endocytosis in toad urinary bladders using fluid phase markers such as horseradish peroxidase (HRP), fluorescent dextran, as well as colloidal gold techniques with applications of transmission electron microscopy (16–17, 19, 23, 32–33). While these studies have contributed toward our understanding of the process of retrieval of water channels as related to endocytosis, little information has been obtained about the time-dependent surface membrane remodeling that occurs following removal of the hormone. In addition, little is known about the phenomena of apical membrane restoration during endocytosis at lower temperatures (26). In our previous studies, we described the behavior of the apical membranes of toad urinary bladder granular epithelia under various time-dependent experimental conditions during exo- and endocytosis (9–10, 21–22, 25). These studies were carried out at laboratory room temperature conditions of 25°C with or without the stimulation by ADH. We observed that during hormone stimulation and exocytosis, ADH-stimulated tissues showed minimal signs of apical membrane remodeling in the form of surface remodeling (21–22, 25). However, ADH-stimulated urinary bladder tissues, following washout, had extensive membrane surface invaginations indicative of surface remodeling. Transmission electron microscopic observations also confirmed the occurrence of large vacuolar compartments within the cytosol concurrent with the apical membrane internalization. To determine if cold temperature could enhance our ability to monitor surface membrane remodeling and water channel endocytosis, we conducted a series of time-dependent experiments of ADH-challenged toad urinary bladder tissues under two different temperatures of 25°C and 15°C.

Endocytosis is a highly regulated physiological process that plays a key role in the uptake of macromolecules from the external environment or in retrieval of membrane components back into the cell (22, 32–36). The process of endocytosis is also found to be temperature sensitive in which decreases in temperature result in an increase in the number and size of endocytic vesicles in thyrocytes and in J774 cells *in vitro* (27). In our studies of toad urinary bladders, we observed a dramatic increase in the number of apical membrane invaginations involving a large population of granular cells both at 25°C and 15°C during a 15-min posthormone washout period in tissues that received ADH. The number of endocytosed invaginations at 25°C was over 44% versus 80% of cells at 15°C, indicating a two-fold increase in the number of invaginations at the lower temperature. The number of invaginations was found to peak at 15 min postwashout as 28% and 33% of the granular cells showed surface invaginations during a 30-min washout recovery period at 25°C and 15°C, respectively. Control tissues during a similar 15-min recovery period at 25°C and 15°C showed no more than 6% and 10% of granular cells with signs of membrane invaginations, respectively. Whereas during 30- and 60-min recovery periods, control tissues showed less than 3% and 6% of granular cells involved at 25°C and 15°C, respectively. These results correlate with our previous studies at 25°C indicating that lowering the temperature to 15°C neither arrested the process of apical membrane restoration nor greatly altered the pattern of apical membrane surface remodeling leading to restoration of the apical membrane to nearly prehormone normal state at 60 min into recovery. However, the restoration process appeared to be somewhat slower at 15°C than at 25°C as observed at 30-min recovery periods.

Previous studies on endocytosis involving toad urinary bladders also indicated an increase in the number and dimensions of the endosomes as determined by HRP and

FITC dextran at 10- and 30-min recovery periods (32, 33). This increase in the number of endosomes was also found to plateau at 30 min into recovery. Zeidel *et al.* (32, 33) also showed that at 30 min and 60 min, the endosomes were larger than 10-min endosomes, whereas the control, unstimulated tissues contained no endosomes. Furthermore, they found no functional water channels at 60 min, presumably inactivated or degraded 60 min following ADH withdrawal (33). Our current and previous SEM studies on endocytosis of the toad urinary bladder apical membranes under various time-dependent experimental conditions (22, 25) also exhibited almost a complete recovery of the apical membranes into normal state after a 60-min washout, with no more than 3% of cells showing surface changes (25). This may indicate that at 60 min following withdrawal of ADH, water channels are largely internalized as endosomes leaving the apical membrane surface virtually water impermeable (25). Eggena (37) has also studied the temperature effect on vasopressin action on toad urinary bladder. He found that although water flow decrased at the lower temperatures, the flow was sustained. He also found that the changes in temperature influenced the conformational state of the water channel.

SEM observations demonstrated an increase in the size of the apical membrane invaginations at different temperatures and at different recovery periods. It was found that there was little difference in the size of invaginations between the control and ADH-stimulated tissues at 25°C during the 15-min postwashout recovery periods but at 30 min at the same temperature, we discovered over 34% increase in the size of invaginations in ADH-stimulated tissues compared to control tissues. We also found increases of over 40% and 146% in the size of invaginations in the ADH-stimulated tissues over control tissues at 25°C and 15°C after 15 and 30 min of the recovery period. Such a dramatic increase in the size of invaginations at the cold temperature was also reported in cultures of thyrocytes and J774 cells (27). However, in the present study, the size of the invaginations was seen to reduce considerably following 60 min of washout of ADH both at 25°C and 15°C, showing the recovery of the granular cells almost to a normal state.

Our observations using SEM and TEM also revealed the collapse of the basolateral membranes due to extreme compression involving many granular cells during recovery periods at 15°C in ADH-stimulated tissues. In addition, we also observed the assembly of a large number of microfilaments associated with basolateral membrane compression at 15°C. At this stage, we have not been able to determine the role of microfilaments and microtubules in apical membrane remodeling. Displacement and compression of the microfilaments, and likely microtubules, along with compressed basolateral membranes may have contributed to the inhibition of water channel endocytosis that was observed at the lower temperature. Taylor *et al.* (38–40) reported an involvement of microtubules in the temperature-dependent inhibition of water flow in colchicine-treated and ADH-stimulated toad urinary bladder tissues. They reported that inhibition of water flow by colchicine was increased by 50% with lowering of the temperature by each 10-degree interval. Pearl and Taylor (41) also demonstrated an inhibition of water flow in ADH-stimulated toad urinary bladder tissues by cytochalasin B, and this inhibition was attributed to loss of microfilament function caused by cytochalasin B. From these observations, one may infer that microtubules and microfilaments have a functional role in the induction of apical membrane internalization associated with endocytosis; however, additional studies are needed.

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