Vacuolar-type H^+-ATPase in mouse bladder epithelium is responsible for urinary acidification

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Abstract The urine in the mouse bladder was found to be acidic, ranging from pH 5.3 to 5.5 in the daytime and pH 6.0 to 6.3 at night. Administration of bafilomycin A_1 or concanamycin A, specific inhibitors of vacuolar-type H^+-ATPase, into bladder lumen caused neutralization of urinary pH at least for 36 h, whereas inhibitors of mitochondrial ATP synthase (F-type H^+-ATPase) or P-type H^+-ATPases did not. Bafilomycin A_1-sensitive proton secretion from isolated inside-out bladder was also observed. Immuno-electron microscopy with antibodies against vacuolar H^+-ATPase revealed that vacuolar-type H^+-ATPase is rich in luminal plasma membrane and endosomes of superficial cells of the bladder epithelium. These results indicate that vacuolar-type H^+-ATPases present in luminal plasma membrane of the superficial epithelial cells secrete protons so as to acidify the urine in mouse bladder.

Key words: Vacuolar-type H^+-ATPase; Bladder; Bafilomycin; Urine

1. Introduction

Vacuolar-type H^+-ATPases (V-ATPases) acidify endomembrane systems, and the resultant acidic pH is essential for various organellar functions such as proteolysis in lysosomes and accumulation of neurotransmitters into synaptic vesicles or microvesicles (reviewed in [1–4]). In some epithelial cells, V-ATPases are abundant at the plasma membrane, extrude protons, and acidify the restricted extracellular space. Examples of the requirement of extracellular acidification are bone resorption in osteoclast and nutrient absorption in insect midgut (reviewed in [5–7]). The mechanism of urinary acidification and ion transport has been studied extensively (reviewed in [5,8,9]). In the proximal and distal urinary tubules of the mammalian kidney, plasma membrane V-ATPase of luminal superficial cells is important for resorption of filtered bicarbonate [5]. Highly regulated systems for resorption of electrolytes have been found in the urinary bladders from amphibia (frog) and reptilia (turtle), counterparts of the proximal and distal tubules of mammalian kidney [5,10,11].

In mammals the bladder is anatomically different from those of amphibia and reptilia. So far, no reports are available on the presence of transport systems for electrolytes. In this study, we investigated whether or not the mouse urinary bladder contains systems for secretion of protons, and found that the V-ATPase at the plasma membrane of superficial epithelium acidifies urine in the bladder.

2. Materials and methods

2.1. Measurement of urinary pH

Female ICR mice (5–7 weeks) were obtained from Charles River Inc. Urine samples (about 10 μl) were collected by gentle massage of the abdomen and their pH values were promptly measured with a micro-pH electrode (model B-112, Horiba Co., Japan). In some experiments, various proton pump inhibitors in 20 μl PBS were injected into the bladder lumen via the urethra [12], and then time courses of the urinary pH were followed.

2.2. Measurement of proton secretion from isolated mouse bladder

Bladder isolated at 2 p.m. (acidic phase of urinary pH) was turned inside-out, washed once with 5 mM HEPES-NaOH (pH 7.2) containing 0.8% NaCl and 0.5 M urea, and placed in the same buffer with or without bafilomycin A_1 (1 μM). Immediately, pH of the medium was measured at 37°C with a pH meter (Benchtop pH/ISE Meter Model 920A, ORION).

2.3. Immunohistochemical detection of V-ATPase

The bladder was frozen in OTC compound (Miles Inc.) at −80°C. Thin sections were treated with antibodies against V-ATPase (800-fold dilution), and stained with peroxidase activity [13]. They were post-fixed with 1% OsO_4, and observed by electron microscopy. Polyclonal antibodies against V-ATPase were raised by successive injections of isolated subunits into albino rabbits [14]. Specificity of the antibodies to the mammalian tissues was extensively characterized previously [15–17].

2.4. Detection of acidic endosomes of isolated superficial cells

Vital staining of acridine orange was used to detect acidic organelles in the living epithelial cells. Briefly, acridine orange (6 μM in PBS) was injected into the bladder lumen via the urethra, and incubated for a further 10 min. Under this condition, acridine orange penetrates through the cell membrane, and accumulates in acidic compartments [18,19]. Then, mice were killed and the bladder was isolated. The epithelium was stripped off, washed with PBS, and observed with a laser scanning microscope (LSM-GB200, Olympus Co.).

3. Results and discussion

3.1. Acidification of urinary pH in the bladder

The urinary pH from mice under conditions with a 12 h light/dark cycle was followed continuously. The pH was found to be acidic and followed a circadian rhythm: pH 5.3–5.5 in the late light period (resting period for mice) and 6.0–6.3 in the dark period (active period for mice) (Fig. 1). Essentially the same results were obtained from 40 mice tested. Since the urinary pH values were significantly lower than that of the

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Abbreviations: V-ATPase, vacuolar-type H^--ATPase; PBS, phosphate-buffered saline

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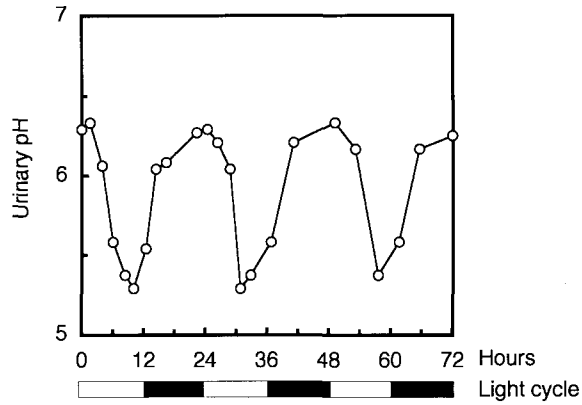


Fig. 1. Daily changes of mouse urinary pH. Urinary pH was followed as described in Section 2. Mice were adjusted to a light (open box, 8.00 a.m. to 8.00 p.m.) and dark (closed box) cycle for one week before use. Values of the urinary pH are the averages for four mice, and the standard deviations were about 7% of the average values.

primary urine (about pH 6.7) [8,9], these results suggest that urine was acidified in the bladder.

To investigate the mechanism of urinary acidification in the bladder, we next tested the effects of inhibitors of various proton pumps. When administrated into the luminal side of the bladder, low concentrations of bafilomycin A_1 or concanamycin A, specific inhibitors of V-ATPase [20–22], inhibited the acidification (Table 1). The inhibition occurred immediately and continued at least for 36 h after a single injection. Nigericin, an H^+/K^+ exchange ionophore, also showed a similar effect, whereas oligomycin, an F-type H^+-ATPase inhibitor [1–3], or vanadate, a P-type ATPase inhibitor [1–3], had no effect (Table 1). These results strongly suggest that V-ATPase is responsible for the urinary acidification in the bladder.

To exclude the possibility that bafilomycin A_1 (or other inhibitors) injected into the bladder was transported to kidney urinary ducts and inhibited the urinary acidification, radiolabeled inulin (0.81 μCi) or tritium water (1 mCi) (20 μl each) was injected into the bladder via the urethra as tracer for V-ATPase inhibitors, and the distributions of the radioactivities in the kidney and bladder were measured. Even after 1 day incubation, radioactivity was detected only in the bladder and essentially no radioactivity was found in the kidney, suggesting strongly that the effects of inhibitors tested are restricted only to the bladder. These results further support that urinary acidification of primary urine occurs in the bladder, and is not directly linked to secretion of protons in the kidney urinary ducts.

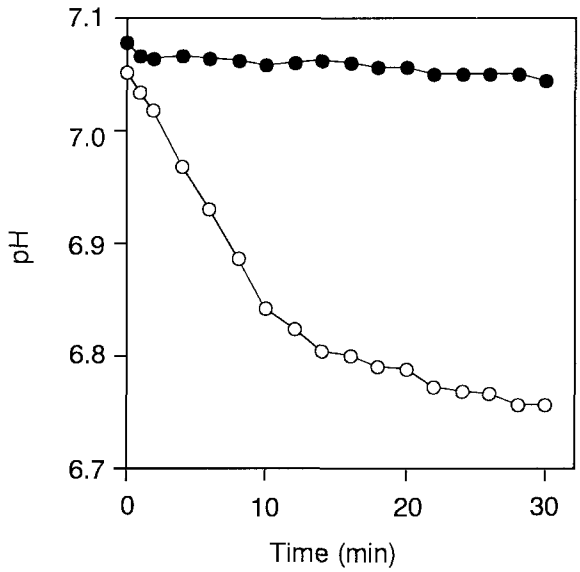
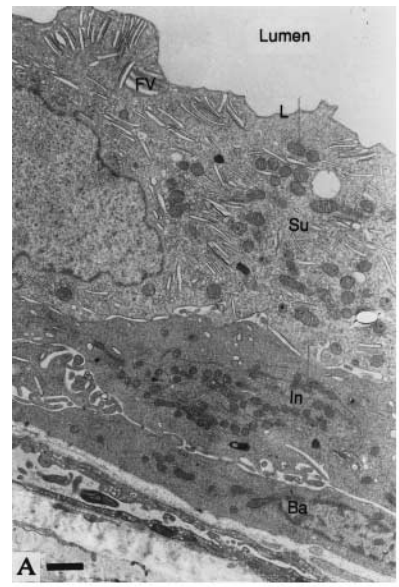


Fig. 2. Proton secretion from isolated bladder. Inside-out bladder was incubated in the medium and its pH change was measured as described in Section 2. Bafilomycin A_1 at 1 μM was included in the medium (closed circle). Inside-out bladder was also incubated without bafilomycin A_1 (open circle).



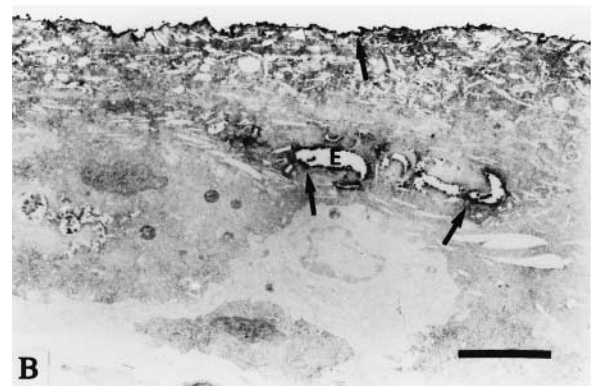


Fig. 3. Localization of V-ATPase in bladder epithelium. A: Conventional electron microscopy of the bladder epithelium. L, luminal membrane of a superficial cell; E, large endosomes; FV, fusiform vacuoles; Su, superficial cell; In, intermediate cell; Ba, basal cell. B: Immuno-electron microscopy using anti-*A* subunit antibodies. Positive areas are shown by arrows. Bar, 5 μm.

More convincing evidence was obtained using isolated inside-out bladder. The inside-out bladder was placed in dilute buffer with or without bafilomycin; the medium pH of the control bladder was acidified rapidly with a maximum velocity of 0.025 pH units/min, whereas that of the bafilomycin-treated bladder showed essentially no acidification (Fig. 2). These results strongly suggest that bafilomycin A_1-sensitive proton secretion actually occurred in the bladder.

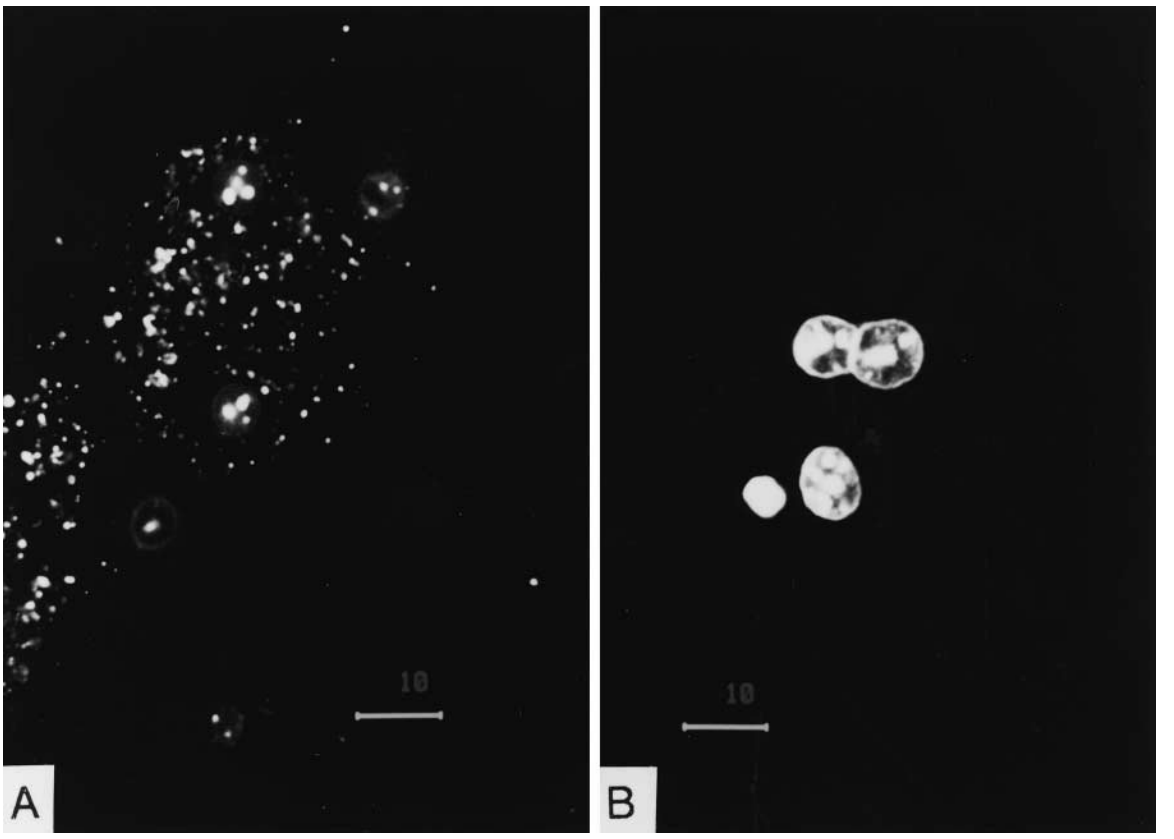


Fig. 4. Detection of acidic organelles in isolated superficial cells. Confocal images of accumulation of acridine orange into living superficial cells in the absence (A) or presence (B) of bafilomycin A_1. Bafilomycin A_1 at 1 μM was injected into the bladder 2 h before acridine orange treatment. Nucleus was also stained with the fluorescent dye upon its binding to DNA. Bar, 10 μm.

3.2. Immunohistochemical detection of V-ATPase

V-ATPases present in plasma membrane may acidify extracellular spaces in osteoclast, collecting ducts in kidney, phagocytes and insect midgut [5–7]. Thus, it is expected that a similar mechanism operates in the urinary acidification in the bladder. Conventional electron microscopic investigation indicated that bladder epithelium is composed of at least three types of cells: superficial, intermediate, and basal cells (Fig. 3A). Immuno-electron microscopy demonstrated that the reactivities with antibodies against subunit *A* of V-ATPase were present in luminal plasma membrane and endosome-like organelles in superficial cells (Fig. 3B). No reactivities were observed in basolateral plasma membranes and fusiform vesicles, a unique vacuolar structure in superficial cells (Fig. 3B), as well as other membrane structures in intermediate or basal cells (data not shown). Similar results were obtained with antibodies against V-ATPase subunits *E* and *I*.

Table 1
Effects of H^+-ATPase inhibitors on urinary pH

Inhibitor	Urinary pH
PBS (control)	5.62 ± 0.24
Bafilomycin A_1 (1 μM)	6.97 ± 0.31
Concanamycin A (1 μM)	6.64 ± 0.32
Nigericin (5 μM)	6.60 ± 0.22
Oligomycin (10 μM)	5.62 ± 0.17
Vanadate (0.5 mM)	5.70 ± 0.28

Listed compounds (20 μl in PBS) were injected into the bladder lumen via the urethra at 8.00 a.m. on day 1 (Fig. 1). After 7 h, urine samples (10 μl) were collected and their pH values were measured. Standard deviations of the pH were obtained from four independent experiments.

Consistent with the localization of V-ATPase, many acidic organelles could be observed in superficial cells (Fig. 4A). The organellar pH increased upon addition of bafilomycin A_1, indicating that the acidic pH is maintained by V-ATPase (Fig. 4B). The numbers and diameters of the acidic organelles were similar to those of the endosomes detected immunologically (Fig. 2). These results suggest that V-ATPase is present in endosomes and luminal plasma membrane in superficial cells. V-ATPase in endosomes may acidify their intravesicular space, while V-ATPase in luminal plasma membranes may secrete protons so as to acidify the urine.

In this study, we demonstrated that V-ATPase in the superficial cell of the mouse urinary bladder is involved in urinary acidification. V-ATPase is rich in endosomes as well as luminal plasma membrane, similar to the V-ATPase localization in other epithelial cells [5–7]. To our knowledge, this is the first report showing the presence of electrolyte transporter in the mammalian urinary bladder. We also observed immunohistochemically that V-ATPase is present in the luminal plasma membrane of superficial cells of human bladder (data not shown). Thus, urinary acidification in the bladder may be a general phenomenon among mammals. It is noteworthy that the urine acidity followed a regular circadian rhythm (Fig. 1). Although the physiological significance of the urinary acidification is unclear at present, acidic pH in the bladder may be involved in the natural defense mechanism against bacterial infection. We are investigating this possibility by measuring

changes of bacterial adhesion to the luminal surface of the isolated bladder.

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References

[1] Nelson, N. (1989) J. Bioenerg. Biomembr. 21, 553–570.
[2] Forgac, M. (1989) Physiol. Rev. 69, 165–196.
[3] Moriyama, Y., Maeda, M. and Futai, M. (1992) J. Exp. Biol. 172, 171–178.
[4] Moriyama, Y., Yamamoto, A., Yamada, H., Tashiro, Y., and Futai, M. (1996) Biol. Chem. Hoppe Seyler 377, 155–165.
[5] Gluck, S. and Nelson, R. (1992) J. Exp. Biol. 172, 205–218.
[6] Baron, R., Bartkiewicz, M., David, P., and Hernando-Sobrino, N. (1994) in: Organellar Proton-ATPases (Nelson, N., Ed.), pp. 49–73. R.G. Landes Company.
[7] Dow, J.A.T. (1994) in: Organellar Proton-ATPases (Nelson, N., Ed.), pp. 76–102. R.G. Landes Company.
[8] Alpern, R.J. (1990) Physiol. Rev. 70, 79–114.
[9] Koeppen, B., Ciebish, G. and Malnic, G. (1985) in: The Kidney: Physiology and Pathophysiology (Seldin, D.W. and Giebish, G., Eds.), pp. 1491–1525. Raven Press, New York.
[10] Steinmetz, P.R. (1986) Am. J. Physiol. 251, F172–F187.
[11] Harvey, B.J., Lacoste, I. and Ehrenfeld, J. (1991) J. Gen. Physiol. 97, 749–776.
[12] Liao, J.K., Tomochika, K., Watanabe, S. and Kanesama, Y. (1992) Microbiol. Immunol. 36, 243–256.
[13] Sadahira, T., Mori, M., Watarai, S. and Vasuda, T. (1988) Blood 72, 42–48.
[14] Moriyama, Y. and Nelson, N. (1989) J. Biol. Chem. 264, 18445–18450.
[15] Moriyama, Y., Yamamoto, A., Yamada, H., Tashiro, Y., Tomochika, K.-I., Takahashi, M., Maeda, M. and Futai, M. (1995) J. Biol. Chem. 270, 11424–11429.
[16] Nakamura, H., Moriyama, Y., Futai, M. and Ozawa, H. (1995) Arch. Histol. Cytol. 57, 535–539.
[17] Sasaki, T., Hong, M.-H., Udagawa, N. and Moriyama, Y. (1994) Cell Tissue Res. 278, 265–271.
[18] Umata, T., Moriyama, Y., Futai, M. and Mekada, E. (1990) J. Biol. Chem. 265, 21940–21945.
[19] Yoshimori, T., Yamamoto, A., Moriyama, Y., Futai, M. and Tashiro, Y. (1991) J. Biol. Chem. 266, 17707–17712.
[20] Bowman, E.J., Siever, A. and Altendorf, K. (1988) Proc. Natl. Acad. Sci. USA 85. 7972–7976.
[21] Hanada, H., Moriyama, Y., Maeda, M. and Futai, M. (1990) Biochem. Biophys. Res. Commun. 170, 873–878.
[22] Ito, K., Kobayashi, T., Moriyama, Y., Toshima, K., Tatsuya, K., Kaiuchi, T., Futai, M., Ploegh, H. and Miwa, K. (1995) J. Antibiot. 48, 488–494.