Ultrastructural Localization of Na\(^+\), K\(^+\)-ATPase in the Exorbital Lacrimal Gland of Rat

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Ultrastructural localization of Na\(^+\), K\(^+\)-ATPase in the exorbital lacrimal gland of rat was investigated quantitatively by protein A-gold technique. Na\(^+\), K\(^+\)-ATPase was purified from the rat kidney, and anti-holo Na\(^+\), K\(^+\)-ATPase antibody was obtained from the rabbit by injecting the purified enzyme. A specific antibody against the α-subunit of Na\(^+\), K\(^+\)-ATPase was affinity purified. Immunoblot analysis revealed that the antibody bound specifically to the α-subunit of Na\(^+\), K\(^+\)-ATPase of the lacrimal gland. Rats were fixed by perfusion with 4% paraformaldehyde containing 1% glutaraldehyde, and the lacrimal glands were embedded in LR White resin. Ultrathin sections were incubated with affinity purified antibody against the α-subunit of Na\(^+\), K\(^+\)-ATPase, and then with protein A-gold complex. The sections were observed under an electron microscope. Light microscopy with silver enhancement procedure revealed that Na\(^+\), K\(^+\)-ATPase was located mainly on the basal region of the cells of intralobular and interlobular ducts. Quantitative immunoelectron microscopic analysis showed that gold particles were found on the basolateral surfaces of the interlobular and intralobular ducts cells and on the basolateral surface of the acinar cells, whereas no significant binding was observed on any part of the apical surfaces of these cells. Labeling density of gold particles was highest on the basolateral surface of the interlobular duct cells, secondarily highest on the basolateral surface of the intralobular duct cells, and lowest on the basolateral surface of the acinar cells. The distribution pattern of Na\(^+\), K\(^+\)-ATPase in the acinar cells and the duct cells suggest that this enzyme may play an important role in primary secretion and in determining the composition of electrolytes in the final secretion, respectively. Invest Ophthalmol Vis Sci 33:196–204, 1992

Materials and Methods

Animals

Adult male Sprague-Dawley rats weighing 150–250 g were used. They were given commercial chow and water ad libitum. All animals were used in accordance with ARVO Resolution on the Use of Animals in Research.

Purification of Na\(^+\), K\(^+\)-ATPase and Antibody Preparation

Purification of Na\(^+\), K\(^+\)-ATPase from the rat kidney and the procedure for immunization of rabbits have been described in detail elsewhere.9 Monospe-
cific antibody against α-subunit of Na\textsuperscript{+}, K\textsuperscript{+-}ATPase was affinity purified by a slight modification of the method of Smith and Fisher\textsuperscript{10} and McDonough et al\textsuperscript{11} as described in previous reports.\textsuperscript{12,13}

**Immunoblot Analysis**

The exorbital lacrimal glands were homogenized and solubilized in 1% sodium dodecyl sulfate (SDS) in a Teflon-glass homogenizer. Insoluble components were removed by centrifugation at 15000 \times g for 15 min. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 8.5% slab gel as described by Laemmli,\textsuperscript{14} and the electrophoresed proteins were transferred to a Millipore GVHP filter (Nihon Millipore Kogyo, Yonezawa, Japan) according to Burnette.\textsuperscript{15} To demonstrate the total proteins transferred to the nitrocellulose sheet, a part of the sheet was stained with 0.5% amido black. The rest of the sheet was incubated overnight with 1% skim milk (Difco, Detroit, MI) in Tris-HCl buffered saline (TBS), pH 7.6 at 4°C. This was incubated with affinity purified antibody against α-subunit of Na\textsuperscript{+}, K\textsuperscript{+-}ATPase (5.0 μg/ml) in TBS containing 0.5% bovine serum albumin (BSA) for 2 h at room temperature, followed by incubation with goat anti-rabbit IgG antibody-horseradish peroxidase conjugate in TBS (6 μg IgG/ml) containing 0.05% Tween 20 (Nacalai Tesque, Inc., Kyoto, Japan). The reacted proteins were visualized with 3,3'-diaminobenzidine tetrahydrochloride color reagent.

**Immunocytochemistry**

For the localization of Na\textsuperscript{+}, K\textsuperscript{+-}ATPase α-subunit in the lacrimal gland, we used a post-embedding method on LR White sections, as described by Roth et al\textsuperscript{16,17} with some modifications. The rats were anesthetized with sodium pentobarbital (Nembutal, 5mg/100 g body weight, intraperitoneal injection). They were perfused from the left ventricle with Hanks' solution, pH 7.4, for 3 min, then fixed by perfusion with 4% paraformaldehyde containing 1% glutaraldehyde in Hanks' solution, pH 7.4, for 10 min and washed with phosphate buffered saline, pH 7.4 (PBS), containing 50 mM ammonium chloride for 5 min. The exorbital lacrimal gland was excised, cut into small pieces, and dehydrated in graded ethanols. It was then embedded in LR White (Bio Rad Microscience Division, Watford, UK) adding 0.5% benzoin methyl ether at −20°C. Polymerization was done at −20°C for 24 h and at room temperature for 24 h in an ultraviolet-ray polymerizer TUV-200 (Dosaka EM Co., Kyoto, Japan).

For the light microscopic observation of Na\textsuperscript{+}, K\textsuperscript{+-}ATPase, 2 μm-thick semithin sections were cut from the LR White embedded lacrimal gland and stained by the protein A-gold silver enhancement procedure, as described by Taatjes et al\textsuperscript{18} with some modifications. Semithin sections were mounted on cover glass strips and incubated with 0.5% BSA in PBS for 5 min to reduce nonspecific binding, then incubated with anti-Na\textsuperscript{+}, K\textsuperscript{+-}ATPase α-subunit antibody (5 μg/ml, with 0.5% BSA in PBS 20 μl), and subsequently with protein A-gold complex (8 nm in diameter; optical density at 525 nm (OD\textsubscript{525}) = 0.08; 100 μl). The sections were washed several times with PBS, then post-fixed by 5% glutaraldehyde in cacodylate buffer, pH 7.4. After washing with distilled water, the glass strips were incubated with 0.6% hydroquinone in 0.1 M citrate buffer, pH 4.0, for 5 min. The glass strips were subsequently incubated with 0.6% hydroquinone, 0.1% silver lactate in the same buffer for 6 min in the dark room, then fixed by a photographic fixer. The sections were slightly counterstained with methyl green to provide better visualization.

For electron microscopic observation, ultrathin sections on collodion-coated nickel grids were floated on drops of 0.5% BSA in PBS for 5 min, incubated with anti-Na\textsuperscript{+}, K\textsuperscript{+-}ATPase α-subunit antibody (5 μg/ml, 20 μl) for 1 h. They were subsequently incubated with protein A-gold (8 nm in diameter, OD\textsubscript{525} = 0.08; 20 μl). After washing several times with cacodylate buffer, pH 7.4, sections were fixed with 5% glutaraldehyde, stained with 2% uranyl acetate for 5 min and stained by the protein A-gold silver enhancement procedure. The rat kidney and lacrimal gland were solubilized in 1% SDS and separated by SDS-PAGE (8.5% slab gel), and transferred to a Millipore GVHP filter. A piece of sheet was stained by amido black (lanes 1, 2). Immunoblot analysis was carried out as described in Materials and Methods (lanes 3, 4). Lanes 1, 3: kidney; lanes 2, 4: exorbital lacrimal gland.

![SDS-PAGE IMMUNOBLOT](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933163/)
Fig. 2. Light microscopic visualization of Na⁺, K⁺-ATPase α-subunit in the lacrimal gland by the protein A-gold silver enhancement procedure. Semithin sections of the lacrimal gland embedded in LR White were incubated with anti-Na⁺, K⁺-ATPase α-subunit antibody (a, b) or with the same concentration of nonimmunized rabbit IgG instead of the antibody as a control experiment (c, d), and processed as described in Materials and Methods. (a, c) Interlobular ducts and the acinus; (b, d) intralobular ducts (arrows) and acinus. Bar = 50 μm.

Reynold's lead citrate (Wako Pure Chemical Industries, Ltd., Osaka, Japan) for a few seconds, then observed with a HU-12 electron microscope (Hitachi, Ltd., Tokyo, Japan).

As a control, the other sections were incubated with the same concentration of IgG from a nonimmunized rabbit instead of the specific antibody, and were then treated by the same procedure as noted above.

Quantitative Analysis

Electron micrographs of the apical and basolateral surface of the interlobular duct cells, intralobular duct cells, and acinar cells were taken randomly and enlarged to a final magnification of × 64,500. The numbers of gold particles on cross-sectional profiles of the apical and basolateral membrane domains were counted, and the lengths of the membranes were measured by a graphic digitizer (Model G-2; Mutoh Industrial, Tokyo, Japan) connected to a personal computer (Sord Model 223 Mark 3; Sord Inc., Tokyo, Japan). In this observation, the basolateral membrane counts included the membrane up to the apical-lateral tight junctions. The specific binding was calculated by subtracting the average number of gold particles per micrometer of membrane (labeling density) of the control from that of the experimental specimens. Three lacrimal glands from three rats were used for each quantitative analysis, and 20 photographs from each of the experimental and control specimens were analyzed.

Determination of Saturation Level of the Antibody for the Immunocytochemical Labeling Assay

To carry out immunocytochemical analysis quantitatively, all the reactions should be carried out at the saturation level of the antibody as reported previously.12,13,19,20 Because the immunocytochemical procedure was slightly modified (resin, fixation, and the diameter of gold particles), the saturation level of the antibody was determined under the present experimental conditions. Ultrathin sections of the rat lacrimal gland (about 2 × 10⁶ μm² in area) were incubated
with 20 μl of increasing concentration of the antibody solution and subsequently with protein A-gold complex, as described above. The labeling density on the basolateral membranes of the interlobular duct cells—where the density of gold particles was highest in the lacrimal gland—was determined.

Results

Immunological Specificity of the Antibody

Immunological specificity of the affinity purified antibody against the α-subunit of the rat kidney Na+, K+-ATPase was tested by immunoblot analysis of the solubilized rat kidney and lacrimal gland (Fig. 1). This antibody bound specifically to the α-subunit of Na+, K+-ATPase in the rat kidney and lacrimal gland (Fig. 1, lanes 3 and 4). The molecular weight of the α-subunit in the lacrimal gland was approximately 100 kD (Fig. 1, lane 4). We used this antibody in all of the following immunocytochemical experiments.

Light Microscopical Localization of Na+, K+-ATPase in the Exorbital Lacrimal Gland

Figures 2a and 2b show the lacrimal gland that was incubated with anti-Na+, K+-ATPase α-subunit antibody and stained by the protein A-gold silver enhancement procedure. The intense precipitates of silver grains were observed in the interlobular ducts (Fig. 2a), while moderate amounts of silver grains are attached to the intralobular ducts at their basal and lateral surfaces (Fig. 2b, arrows). In the acinus, weak Na+, K+-ATPase immunoreactivity was detected along the basal region (Figs. 2a, 2b). When the sections were incubated with nonimmunized rabbit IgG as a control experiment, silver grains were barely observed (Figs. 2c, 2d).

Electron Microscopical Localization of Na+, K+-ATPase in the Exorbital Lacrimal Gland

Figures 3–11 show electron micrographs of the exorbital lacrimal gland, which was embedded in LR White. When sections were incubated with antibody against the α-subunit of Na+, K+-ATPase, many gold particles bound to the basal and lateral plasma membranes in interlobular and intralobular duct cells (Figs. 3, 4 and 6, 7, respectively). In acinar cells, a small number of gold particles was observed on the basolateral plasma membrane of the interlobular duct cells (Fig. 10, arrowheads). The apical plasma membranes of these cells were barely labeled (Figs. 3, 6, 9). In the control experiment incubated with nonimmunized IgG, gold particles were scarcely observed (Figs. 5, 8, 11).

Saturation of Antibody Binding in the Quantitative Immunocytochemical Assay

Figure 12 shows the saturation curve of the antibody to the basolateral surface of the interlobular duct cells. The density of gold particles reached a saturation level when the ultrathin sections were incubated with the antibody at concentrations of more than 2.5 μg/ml. We always incubated the ultrathin sections of the rat exorbital lacrimal gland at a concentration of 5 μg/ml. Thus, in the present experiment, the immunocytochemical reaction was carried out at a saturated concentration of the antibody, indicating that the antibody might bind to almost all of the accessible epitopes of the α-subunit molecules on the ultrathin sections of the lacrimal gland.

Quantitative Analysis of Gold Particles on the Lacrimal Gland

Because the immunocytochemical analyses were always carried out at a saturation level, comparing the distribution of Na+, K+-ATPase α-subunit quantitatively by counting the number of gold particles was possible. The average number of gold particles per micrometer of each domain of the plasma membrane is shown in Table 1. The specific binding was calculated by subtracting the density of control specimens from the density of experimental ones. Student’s paired t-test indicates that the density of gold particles in experimental specimens incubated with specific antibody is statistically significant by above control specimens incubated with nonimmunized IgG on the basolateral plasma membrane of the interlobular duct cells, intralobular duct cells, and acinar cells. However, no significant binding was found on the apical plasma membranes of these cells. The density of gold particles was highest on the basolateral plasma membrane of the interlobular duct cells (about 2.0/μm), secondarily highest on that of the intralobular duct cells (about 1.3/μm), and lowest on that of the acinar cells (about 0.4/μm), as shown in Table 1.

Discussion

The rat has exorbital and infraorbital lacrimal glands. They share the same embryological origin and are histologically analogous to each other. In the present study, we used the exorbital lacrimal gland.

The fluid secretion of the lacrimal gland depends on Na+ and is inhibited by ouabain, a Na+, K+-ATPase inhibitor. This suggests that Na+, K+-ATPase plays a major role in lacrimal gland secretion. Thus, determination of the exact and quantitative localization of Na+, K+-ATPase in the lacrimal gland is very important. In this immunocytochemical study, we used an affinity purified antibody against the α-sub-
unit of Na\(^+\), K\(^+\)-ATPase, which specifically bound to the \(\alpha\)-subunit of Na\(^+\), K\(^+\)-ATPase in the exorbital lacrimal gland of rat.

Localization of Na\(^+\), K\(^+\)-ATPase in the lacrimal gland has been studied by various techniques, such as [\(^3\)H]-ouabain autoradiography, subcellular fractionation analysis, and histochemical and immunocytochemical methods. With autoradiography using [\(^3\)H]-ouabain, Dartt et al\(^2\) reported that a high density of Na\(^+\), K\(^+\)-ATPase was on the basolateral surface of the duct system of rabbit lacrimal glands, whereas a very low density was found on the basolateral surface of the acinar cells. However, Ueno et al\(^6\) reported that this enzyme was located on the basolateral surface of the duct cells of rat lacrimal gland but not on the acinar cells. They detected ouabain-sensitive, potassium-dependent p-nitrophenylphosphatase activity. A similar result was obtained in rat and mouse lacrimal glands by the light microscopic observation of Winston et al\(^8\), who used an antibody against the \(\alpha\)-subunit of this enzyme and an immunoperoxidase technique. By subcellular fractionation of rat lacrimal gland, Mircheff et al\(^4,5\) present evidence that while Na\(^+\), K\(^+\)-ATPase is expressed at the highest specific activities in the basolateral plasma membranes\(^4\), and in the membranes derived from the Golgi complex.\(^5\) According to immunocytochemical study by Wood and Mircheff\(^7\) using antibody against holo-Na\(^+\), K\(^+\)-ATPase, immunofluorescence was detected not only in the ducts but also in the acinus of rat lacrimal gland. The fluorescence of the apical side of the acinus was stronger than the basal side. However, the localization of Na\(^+\), K\(^+\)-ATPase in the acinus was criticized by Winston et al\(^8\).

Thus, abundant localization of Na\(^+\), K\(^+\)-ATPase in the basolateral membranes of the duct cells has been well established, but the presence of this enzyme on the apical surface of the acinar cells in the lacrimal gland has not been settled.

We determined the distribution of Na\(^+\), K\(^+\)-ATPase in the rat exorbital lacrimal gland by protein A-gold technique, using affinity purified antibody against the \(\alpha\)-subunit. Immunoelectron microscopy is based on the high specificity of antigen-antibody reaction and has high sensitivity and high resolution at the electron microscopic level. Moreover, use of the post-embedding immunogold technique on LR White sections ensures equal accessibility of the antibody to the antigenic sites on the ultrathin sections. Our quantitative analysis showed that Na\(^+\), K\(^+\)-ATPase exists on the basolateral surfaces of the duct cells and acinar cells of rat lacrimal gland. The labeling density in the basolateral surface of the interlobular duct cells and of

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**Table 1. Labeling density of gold particles on various cell surfaces of rat lacrimal gland**

<table>
<thead>
<tr>
<th>Cells and cell surfaces</th>
<th>Antibody to Na(^+), K(^+)-ATPase (\alpha)-subunit</th>
<th>Nonimmunized IgG</th>
<th>Specific binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interlobular duct cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apical</td>
<td>0.032 ± 0.017</td>
<td>0.022 ± 0.005</td>
<td>0.009 ± 0.012§</td>
</tr>
<tr>
<td>Basolateral</td>
<td>1.976 ± 0.277</td>
<td>0.017 ± 0.004</td>
<td>1.939 ± 0.273†</td>
</tr>
<tr>
<td>Intralobular duct cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apical</td>
<td>0.041 ± 0.029</td>
<td>0.030 ± 0.036</td>
<td>0.013 ± 0.051§</td>
</tr>
<tr>
<td>Basolateral</td>
<td>1.349 ± 0.478</td>
<td>0.015 ± 0.010</td>
<td>1.334 ± 0.474§</td>
</tr>
<tr>
<td>Acinar cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apical</td>
<td>0.012 ± 0.020</td>
<td>0.017 ± 0.019</td>
<td>-0.005 ± 0.026§</td>
</tr>
<tr>
<td>Basolateral</td>
<td>0.426 ± 0.050</td>
<td>0.011 ± 0.018</td>
<td>0.415 ± 0.040†</td>
</tr>
</tbody>
</table>

* Measured on 20 photographs of the apical and basolateral plasma membranes of three lacrimal glands from three rats, and presented as the mean standard deviation.
† Statistically significant, \(P < 0.005\).
‡ Statistically significant, \(P < 0.025\).
§ Statistically not significant, \(P \approx 0.05\).
Figs. 6-8. Electron micrographs of the intralobular duct cells. Fig. 6. The apical and lateral surfaces of intralobular duct cells. Gold particles are scarcely observed on the apical surface. The lateral surfaces are labeled with gold particles. Original magnification x64,500; bar = 0.5 µm. L, lumen. Fig. 7. The basolateral surface of intralobular duct cells is stained with gold particles. Original magnification x64,500; bar = 0.5 µm. Fig. 8. Control experiment for Figure 7. Few gold particles are observed in the control experiment. Original magnification x64,500; bar = 0.5 µm.
the intralobular cells was about 5 and 3 times higher than that in the basolateral surface of the acinar cells, respectively. No significant binding of gold particles was observed on the apical surfaces of the acinar cells, intralobular cells, or interlobular cells (Table 1). The basolateral surfaces of the duct cells have a 3–5 times higher concentration of Na⁺, K⁺-ATPase. Furthermore, the basolateral surfaces of duct cells have a much larger surface area than those of acinar cells because of the well-developed basal infoldings. Therefore, the average content of Na⁺, K⁺-ATPase in duct cell might be much larger than that in acinar cell. This difference may make detecting Na⁺, K⁺-ATPase in the acinar cells by light microscopic immunocytochemistry difficult. We could not detect any apical labeling of Na⁺, K⁺-ATPase in the lacrimal gland. We suggest that the reported apical labeling of acinar cells in lacrimal gland might be a result of some cross-reactive molecules other than Na⁺, K⁺-ATPase.22

In the rat lacrimal gland, the primary fluid secreted by the acinus is blood plasma-like in ionic composition. It contains almost the same concentration of Na⁺, Cl⁻, K⁺ as the blood plasma. The final secretion collected from the main duct, however, contains much a higher K⁺ concentration, whereas Na⁺ concentration is almost the same as that in the primary secretion.1 It has been suggested that a significant amount of K⁺ must be secreted by the ductal cells.2 Na⁺, K⁺-ATPase, Na⁺/H⁺, and Cl⁻/HCO₃⁻ antiporters; K⁺ channels in the basolateral membranes; and Cl⁻-channels in the apical plasma membranes may make a system for secreting fluid into the luminal space.23 The localization of a large amount of Na⁺, K⁺-ATPase on the basolateral plasma membrane of the duct cells may play a role in K⁺ secretion from the basal to the luminal side in duct system, as suggested by Dartt et al.2 Thus, Na⁺, K⁺-ATPase in the lacrimal gland may be responsible for isotonic secretion in the acinar cells and K⁺ secretion in the duct cells.

**Fig. 12.** Saturation curve for the binding of gold particles to the basolateral plasma membranes of the interlobular duct cells of lacrimal gland. The ultrathin sections of the rat exorbital lacrimal gland (approximately 2 × 10⁴ µm² in area) were incubated with 20 µl of increasing concentration of antibody against the α-subunit of Na⁺, K⁺-ATPase, then stained with protein A-gold complex. The particle density on the basolateral membrane of the interlobular duct cells was determined. The density of gold particles reached a saturation level when the ultrathin sections were incubated with the antibody at concentrations of more than 2.5 µg/ml.
Key words: lacrimal gland, Na+, K+-ATPase, silver enhancement procedure, immunocytochemistry, protein A-gold procedure

References