[3H]Ouabain Localization of Na-K ATPase in the Epithelium of Rabbit Ciliary Body Pars Plicata

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The secretion of the aqueous humor has been proposed to occur as the result of active Na+ transport by a ouabain-sensitive Na-K ATPase. We have examined the localization of this enzyme in the epithelium of rabbit ciliary body pars plicata using [3H]ouabain autoradiography. Single ciliary processes were isolated and incubated in Ringer containing [3H]ouabain. Processes were then rapidly frozen, freeze-dried, sectioned and exposed for autoradiography. In the light microscope, silver grains were found predominantly over the nonpigmented epithelial cells. In the electron microscope, grains could be localized for the most part to the interdigitations of the nonpigmented cell basolateral membrane. Label could also be observed at a much lower density above other membranes and above the pigmented and nonpigmented cell cytoplasm. No label was found in sections of control tissue which had been incubated in [3H]ouabain with an excess of cold ouabain. To show that the [3H]ouabain had free access to all of the membrane surfaces within the epithelium, in parallel experiments we incubated isolated processes in horseradish peroxidase. Our experiments suggest that most of the active Na+ transport in ciliary body epithelium occurs across the basolateral membrane of nonpigmented cells into the posterior chamber. Furthermore, the placement of the Na-K ATPase within the narrow membrane infoldings of the interdigitations is consistent with a role for this enzyme in water transport and the production of the aqueous. Invest Ophthalmol Vis Sci 29:606-614, 1988

The aqueous humor is produced by an epithelial cell layer which covers the surface of the ciliary body at the margin of the eye adjacent to the lens. Although the mechanism of aqueous production is not completely understood, there is reason to believe that much of the aqueous may be produced by active transport.1 Water transport in many epithelial tissues has been shown to be produced by the active transport of Na by a Na-K-dependent, ouabain-sensitive ATPase.2 The ciliary body contains a high concentration of this enzyme3 and aqueous inflow can be reduced by inhibitors of the Na-K ATPase such as ouabain4,5 and vanadate.6 Furthermore, the luminal membrane of the nonpigmented cells has a high hydraulic conductivity and, as in other water-trans
binding in epithelia has been shown to be quantitatively correlated with the inhibition of Na transport.\textsuperscript{23,24} A preliminary report of this work was presented at the annual meeting of the Association for Research in Vision and Ophthalmology.\textsuperscript{30}

Materials and Methods

Pigmented rabbits (2 kg) were sacrificed with a lethal injection of chloral hydrate or of sodium pentobarbital. This procedure accorded with the ARVO Resolution on the Use of Animals in Research. The eyes were rapidly enucleated and hemisected posterior to the ora serrata. The anterior half was placed on a slide warmer in a petri dish filled with modified Ringer's solution (MRS) consisting of 126 mM NaCl, 3.82 mM KCl, 12 mM NaHCO\textsubscript{3}, 0.311 mM CaCl\textsubscript{2}, 0.63 mM MgSO\textsubscript{4}, 5 mM glucose, and 0.1% phenol red adjusted to pH 7.1 by equilibration with 95% O\textsubscript{2}/5% CO\textsubscript{2} at 37°C. Secondary and primary ciliary processes were carefully excised from the ciliary body under a Zeiss Op-Mi stereo microscope (Carl Zeiss, Oberkochen, West Germany) and gently transferred with a Pasteur pipette to one of two small cylindrical cages 8 mm long and 10 mm in diameter, terminated at either end with wire mesh. These cages were placed in a vial also containing MRS at 37°C. Each cage contained about five to ten ciliary processes. One or two ciliary processes were also removed from each eye and placed in a chamber for volume regulation measurements.\textsuperscript{7} These processes were perfused in MRS of the same composition as for the [\textsuperscript{3}H]ouabain incubations and could be demonstrated to show a component of volume regulation which was blocked by ouabain.\textsuperscript{7} Thus it is likely that the processes used in our experiments contained an active Na-K ATPase.

Radioactive Labelling With [\textsuperscript{3}H]Ouabain

The labeling procedures were for the most part similar to those previously described by Bok\textsuperscript{31} and by Stirling and Lee.\textsuperscript{28} For experimental samples, the isolated ciliary processes in the cages were preincubated in MRS for 15 min. The cages were then incubated for 90 min in MRS containing 2 X 10\textsuperscript{-6} M [\textsuperscript{3}H]ouabain (NEN Research Products, Boston, MA). For control samples, the cages were preincubated for 15 min in MRS containing 10\textsuperscript{-4} M nonradioactive ouabain (Sigma Chemical Co., St. Louis, MO). They were then transferred to medium containing 10\textsuperscript{-4} M nonradioactive ouabain together with 2 X 10\textsuperscript{-6} M [\textsuperscript{3}H]ouabain and incubated for 90 min. All solutions were at 37°C and were equilibrated with 95% O\textsubscript{2}/5% CO\textsubscript{2} at pH 7.1. After the incubations, the samples were washed with fresh MRS twice for 5 min each and rapidly frozen.

Rapid Freezing

Methods for rapid freezing were similar to those of Usukura, Akahori, Takahashi and Yamada.\textsuperscript{32} Ciliary processes were placed on filter paper moistened with MRS in the specimen holder of an RF-20 Quick Specimen Freezer (Eiko Engineering Ltd., Sakado Mito Ibaraki, Japan) and immediately washed again with a few drops of fresh MRS. A small piece of filter paper was touched to the edge of the specimen holder to absorb excess solution. The specimen holder was then fastened onto the plunger. Specimens were quickly frozen by pressing them onto a polished copper surface, cooled with liquid helium. Frozen specimens were stored temporarily in liquid nitrogen.

Freeze-Drying and Vapor Fixation

A cubic bronze block (85 mm\textsuperscript{3}) with a cavity in the center (4 cm deep X 5 cm long X 1.2 cm wide) was employed for freeze-drying of samples. Frozen specimens were transferred into the cavity of this block, which had been prechilled in liquid nitrogen prior to use. The cold block containing the specimens was immediately placed in a vacuum evaporator and kept for about 48 hr at 3-7 X 10\textsuperscript{-6} mm Hg. Since the bronze block had a large heat capacity, the specimens in it were warmed up to room temperature with a slow time course. Dried specimens were carefully removed to a glass vial which was saturated with OsO\textsubscript{4} vapor, and the tissue was fixed for 30 min.

Autoradiography

Dried and fixed samples were infiltrated in a vacuum oven (10\textsuperscript{-7} torr) with Spurr's epoxy resin mixture (Polysciences, Inc., Warrenton, PA) following the manufacturer's protocol for the rapid cure method. For light microscopic autoradiography, semithin sections (1 \mu m thick) were collected on glass slides and coated by dipping in Kodak NTB-2 emulsion (Eastman Kodak Co., Rochester, NY) at 40°C. The sections were exposed for 7 days, developed in Dektol (Eastman Kodak), and stained with a 1% solution of Toluidine Blue in 1% sodium borate.

For electron microscopic autoradiography, ultrathin sections were cut and collected on glass slides coated with 2% celloidin. After staining with uranyl acetate and lead citrate, sections were covered with a thin carbon film and then coated by dipping in Ilford L4 emulsion (Polysciences, Inc.) diluted 4:1 with distilled water at 40°C. The autoradiograms were exposed for 4 months and developed in phenidion (K
and K Rare Chemicals, Plainview, NY). These procedures are similar to those previously described.\textsuperscript{33}

**Labeling With Horseradish Peroxidase (HRP)**

To ascertain the degree of penetration of \(^{3}H\)ouabain in our experiments, we incubated isolated ciliary processes for 90 min in MRS containing 0.5 mg/ml HRP (Type VI, Sigma Chemicals Co., St. Louis, MO) under conditions identical to those used for the radioactive ouabain labelling experiments. Specimens were subsequently washed with MRS twice for 5 min each and then fixed with 2.5\% glutaraldehyde plus 2\% paraformaldehyde in phosphate buffer (pH 7.4). Fixed tissue was washed with the phosphate buffer and then placed in fresh phosphate buffer containing 0.05\% diaminobenzidine (Polysciences Inc.) for 15 min. Five microliters of 30\% \(H_{2}O_{2}\) was added to this solution (final concentration, 0.015\%), and the tissue was further incubated in the \(H_{2}O_{2}\)-diaminobenzidine mixture for 5 min. After washing two to three times with fresh phosphate buffer, specimens were dehydrated with an ascending series of ethanol concentrations and embedded in Polybed 812 (Polysciences Inc.). Sections were examined without osmication or any additional staining.

**Results**

The epithelium of the ciliary body consists of two cell layers: a pigmented layer whose basal membrane is adjacent to the fenestrated capillaries of the stroma, and a nonpigmented layer whose basal membrane faces the posterior chamber. The apical membranes of these two cell layers are juxtaposed and interconnected by an extensive network of intercellular junctions.\textsuperscript{34} When single ciliary processes were incubated with \(^{3}H\)ouabain, fast-frozen, freeze-dried, exposed to photographic emulsion, developed, stained with Toluidine Blue and examined in the light microscope, silver grains could be detected primarily above the nonpigmented epithelial cells (Fig. 1A). The density of grains seemed highest in the region where staining with Toluidine Blue appeared lightest. As we shall show below, this region probably corresponds to the basal and lateral interdigitations of the nonpigmented cells. Silver grains could also be detected at a somewhat lower frequency above the pigmented cell layer, where they could be easily distinguished from pigment granules by their size, color and plane of focus. Sections from control experiments, in which ciliary processes were preincubated in nonradioactive ouabain and then incubated in \(^{3}H\)ouabain with an
Fig. 2. Low-power electron microscopic autoradiographs of adult pigmented rabbit ciliary body process incubated in Ringer containing $[^3H]$ouabain. A and B are both from experimental samples incubated as in Figure 1. Grains are present above membranes of lateral and basal interdigitations of nonpigmented cells. Grains are less frequently observed over other membranes and cytoplasm of nonpigmented cells or in the pigmented cell layer. See text. Magnifications: A, $\times$7000; B, $\times$8300.
Fig. 3. High-power electron microscopic autoradiograph of adult pigmented rabbit ciliary body process incubated in Ringer containing [3H]ouabain. Autoradiograph is from an experimental sample incubated as in Figure 1. Magnification ×13,000.
excess of nonradioactive ouabain, showed no significant labeling (Fig. 1B).

**Electron Microscopic Autoradiography**

In Figure 2, we show low-power electron micrographs of thin sections of the ciliary body epithelium. Although our fast-freezing and freeze-drying procedure resulted in some structural damage to the cell cytoplasm and nucleus, probably as the result of ice crystal formation, the overall ultrastructure of the epithelial cells was well preserved. Basal and lateral interdigitations of nonpigmented cells were largely intact, and organelles such as mitochondria and pigment granules could be easily identified.

Silver grains were observed with highest frequency over the interdigitating membranes of the nonpigmented cells. Silver grains were rarely observed above the basal membrane of the nonpigmented cells outside of the interdigitations. Some silver grains could be observed above the nonpigmented cell cytoplasm, as well as at the region of juncture of the apical membranes of the two epithelial cell layers. Grains could also be seen within the pigmented cell layer, over the cytoplasm and basal membrane. The density of grains in these areas was lower than in the nonpigmented cell interdigitations but higher than in extracellular space or stroma.

A higher-power electron microscopic autoradiogram of ciliary body epithelium is given in Figure 3. There is again a dense accumulation of silver grains above the basal and lateral interdigitations of the nonpigmented cells. The density of silver grains elsewhere within the two epithelial cell layers is considerably lower.

**Incubation of Ciliary Processes in HRP**

In order to show that label in our experiments had access to all of the membrane surfaces of the epithelium, we incubated ciliary processes in Ringer containing horseradish peroxidase (HRP) under conditions identical to those used for the $[^{3}H]ouabain$ experiments. HRP is a 40,000 dalton globular protein often used in delineating extracellular space and is considerably larger than ouabain (molecular weight, 729).

In Figure 4, we show a light micrograph of a section of a ciliary process incubated in HRP, fixed and then treated with diaminobenzidine. The presence of HRP can be inferred from the dark deposit of the diaminobenzidine polymer. HRP can be seen to have filled the stroma of the ciliary process. No HRP was present within the nonpigmented cell layer, probably because the HRP in this layer was directly exposed to the extracellular medium and was washed out during fixation and embedding.

Electron micrographs of processes incubated in HRP are given in Figure 5. In Figure 5A, we show a low-power micrograph of the tip of a ciliary process. Since this section was not osmicated or counterstained, the HRP can be recognized from the somewhat higher electron density of the diaminobenzidine polymer. HRP is present within the stroma and can also be localized to the extracellular spaces around the basal membrane of the pigmented cells and along the lateral surfaces of these cells all the way up to their apical membranes. Although little HRP is present within the nonpigmented layer, one nonpigmented cell can be seen to be full of label (arrow), probably as the result of damage to this cell during the incubation.

The distribution of HRP around the pigmented cells can be seen more clearly in the higher-power electron micrograph of Figure 5B. HRP can be seen to have invaded all of the extracellular space between the pigmented cells (small arrows). HRP is also present in the space between the pigmented and nonpigmented cell layers, including the ciliary channels (large arrow) formed by the microvillar extensions of the epithelial cell apical membranes. These experiments show that HRP enters the stroma of isolated ciliary processes during our incubations and penetrates the pigmented cell layer up to the junctional...
Fig. 5. Electron microscopy of adult pigmented rabbit ciliary process incubated for 90 min in Ringer containing HRP as in Figure 4. Sections are unstained. (A) Low power electron micrograph. Note dense deposit of HRP within the stroma of ciliary process. HRP-containing cell within nonpigmented cell layer (arrow) was probably damaged during incubation. Magnification ×4700. (B) Higher power electron micrograph. Note accumulation of HRP within the spaces between pigmented cells (small arrows) and within the ciliary channels between the pigmented and nonpigmented cell apical membranes (large arrows). Magnification ×16,000.
complexes in a manner similar to that which occurs when HRP is injected into the circulation of an intact animal.36-39

Discussion

Our experiments show that when single isolated processes of ciliary body from adult pigmented rabbits are incubated in [3H]ouabain, most of the silver grains are localized above the basal and lateral interdigitations of the nonpigmented cell basolateral membrane. Some grains are found above other membranes within the ciliary body epithelium but at a much lower density. Grains are also found above the cytoplasm of the pigmented and nonpigmented cells. Since ouabain is not membrane-permeant, it is unlikely that these grains could have resulted from the entry of ouabain into the cells during the incubation. It seems more likely that some of the label was translocated during embedding and sectioning.

In previous attempts to localize the Na-K ATPase using the Wachstein-Meisel modification of Gomori's lead phosphate technique, label was also found over the basal and lateral interdigitations9-13,20 and, in some studies, between the apical membranes of the pigmented and nonpigmented cells at the border between the two epithelial cell layers.12,13 These findings are difficult to interpret. On the one hand, it is of considerable interest that the interdigitations, which showed most of the [3H]ouabain binding, also showed deposition of Pb3(PO4)2 precipitate in spite of the difficulties inherent in the Wachstein-Meisel method.16-20 On the other hand, the apical membranes of the pigmented and nonpigmented cells, where Pb3(PO4)2 has also been found, revealed no pronounced localization of [3H]ouabain. Precipitate in this region may have been produced by the activity of another ATPase or by some other lack of specificity of the lead phosphate technique.17,18

Our results show that most of the Na-K ATPase within the processes of the pars plicata appears to be localized to the membranes of the basal and lateral interdigitations. Since we have only examined [3H]ouabain binding within the ciliary processes, we cannot exclude the possibility that there are regional differences in Na-K ATPase distribution between the pars plicata and pars plana. Furthermore, though the density of silver grains is highest above the interdigitations, it is possible that Na-K ATPase is present at a lower density elsewhere within the ciliary body epithelium, in particular at the basolateral membrane of the pigmented cells.14,40 In spite of these reservations, it seems reasonable to conclude that most of the active Na" transport in ciliary body epithelium occurs across the basolateral membrane of the nonpigmented cells into the posterior chamber. The placement of the Na-K ATPase within the highly infolded membranes of the interdigitations is consistent with a role for this enzyme in the transport of water34 and secretion of the aqueous.1,42

Key words: ciliary body, epithelium, Na-K ATPase, [3H]ouabain localization, water transport, aqueous humor, glaucoma

References