Cellular Distribution and Differential Gene Expression of the Three α Subunit Isoforms of the Na,K-ATPase in the Ocular Ciliary Epithelium*

(Received for publication, September 27, 1989)

Sikha Ghosh†, Anna C. Freitag‡§, Pablo Martin-Vasallo¶, and Miguel Coca-Prados||

From the Departments of Ophthalmology and Cell Biology, Yale University School of Medicine, New Haven, Connecticut 06510

We have investigated the localization and pattern of expression of the three α subunit isoforms of Na,K-ATPase in the transporting ciliary epithelium of the bovine eye. Using specific cDNA probes and antisera to the α1, α2, and α3 isoforms of Na,K-ATPase, we demonstrated that mRNAs and polypeptides for the three distinct forms of the Na,K-ATPase α subunit (α1, α2, and α3) were expressed in the ciliary epithelium in vivo. Immunochemical localization of the three α isoforms of Na,K-ATPase in two ultrastructurally different regions of the ciliary epithelium (namely, the pars plicata and pars plana) revealed that the three α isoforms of Na,K-ATPase were distributed in a distinct fashion in the basolateral plasma membrane domains of nonpigmented (NPE) and pigmented (PE) cells. The NPE cells in the pars plicata showed an immunoreactive signal to all the three α isoforms; in the pars plana, they showed immunoreactive signals only for the α1 and α2 isoforms but not for α3. The PE cells, in both the pars plana and pars plicata regions, showed an immunoreactive signal only for the α1 isoform; immunoreactive signals were not detected for α2 and α3. To verify the differential immunostaining patterns of NPE and PE cells, specific antibodies for each of the three α subunit isoforms of Na,K-ATPase were applied to immunohistochemical sections of NPE and PE tissue. Our results indicate that α1, α2, and α3 polypeptides were present in microsomal fractions of NPE cells of the pars plicata and pars plana and that the α1 polypeptide was the only polypeptide present in the PE cells from both regions of the ciliary epithelium. These results also revealed that the α3 isoform epitope recognized by the monoclonal antibody McD-X3.1 in the pars plicata is not readily accessible in the pars plana. A cell line was established from the ciliary epithelium of a bovine eye by viral transformation with simian virus 40. In culture, this cell line expressed all three α isoforms at the mRNA and polypeptide levels, suggesting that the line may have derived from the NPE layer.

*This work was supported by National Institutes of Health Grant EY-04873. Additional support was from Visual Core Grant EY-00785, The Connecticut Lions Eye Research Foundation, Inc., Research to Prevent Blindness, Inc., and Alcon Laboratories. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
† Present address: Facultad de Medicina, Departamento de Bioquímica y Biología Molecular, Universidad de la Laguna, Tenerife, Spain.
‡ To whom all correspondence should be addressed.

The aqueous humor is a fluid composed of water, ions, and proteins, secreted by the ciliary epithelium in the posterior chamber of the mammalian eye. It flows into the anterior chamber and leaves the eye through the trabecular meshwork and Schlemms channel at the sclero-corneal angle. Several functions have been attributed to the aqueous humor, among them: (a) it provides nutrients to the avascularized tissues of the anterior segment of the eye such as the cornea and the lens; (b) it creates an intraocular pressure that maintains the proper curvature to the cornea. The anatomical complexity of the ciliary epithelium has hampered progress in unraveling how aqueous humor formation is regulated across the double ciliary epithelial layer (1). One ion pump required for active transport of aqueous humor by the ciliary epithelium is Na,K-ATPase. Na,K-ATPase is a transmembrane protein composed of two different polypeptides: the catalytic α subunit and the β subunit (for review, see Ref. 2). The sodium pump exchanges 3Na+ outward for 2K+ inward into the cell. Ultrastructural studies on the ciliary epithelium of the mammalian eye have revealed regional and cellular differences that have been suggested possibly to reflect important differences in the transporting activity of the nonpigmented (NPE) and pigmented (PE) cells (3, 4). Recent evidence has suggested that the bovine NPE and PE cells differ in the relative levels of two distinct isoforms (α and α+) of Na,K-ATPase (5). The NPE cells are thought to provide the bulk of the ion transporting capacity, containing 2–3-fold more Na,K-ATPase enzymatic activity than the PE cells (6, 7). Three isoforms of the catalytic subunit of Na,K-ATPase have been identified so far by recombinant DNA experiments. These have been designated α1, α2, and α3, respectively (8–12). In rat, each of the α subunit isoforms was shown to be expressed in a tissuespecific and developmentally regulated fashion (11–14). Although the α1 form of the enzyme was found to be expressed in all the tissues tested, the α2 and α3 forms were expressed predominantly in the brain (15). The functional significance of this diversity has not been clearly explained. The availability of cDNA probes and monospecific antibodies for the three α isoforms of Na,K-ATPase has led us to reexamine the expression of Na,K-ATPase α subunit isoforms in the bovine ciliary epithelium.

In this paper we describe the cellular distribution and expression of the Na,K-ATPase α subunit isoforms in two structurally different regions of the ciliary epithelium: the pars plicata and the pars plana. To facilitate the study of the transport kinetics of the α isoforms of Na,K-ATPase in the ciliary epithelial cells, we have established and characterized a cell line derived from the bovine ciliary epithelium.

The abbreviations used are: NPE, nonpigmented epithelium; PE, pigmented epithelium; SS, side scatter; FLS, forward light scatter; PB, phosphate buffer; SDS, sodium dodecyl sulfate; kb, kilobase(s).
Eye Tissue—Bovine eyes from 2-5-day-old calves were obtained from a local slaughterhouse, and the ciliary epithelium was dissected with the aid of a binocular dissection microscope (16). The ciliary epithelium of the pars plicata was subdissected in two regions: the pars plicata and the pars plana region. The former projects freely for a distance of approximately 2-3 mm over the iris, the so-called operculum or corpora nigra. The pars plana region extends from the pars plicata region to the region in which the sensory layers of the retina start, the so-called ora serrata (Fig. 1).

Flow Cytometric Analysis and Sorting of NPE and PE Cells—Ciliary epithelial cells from the pars plicata or pars plana regions were dissociated into single cells (NPE and PE) as described previously (17) and sorted in a Becton-Dickinson fluorescence-activated cell sorter (FACS 440) equipped with a 5-watt argon ion laser operating at 488 nm with an output of 400 mWatts. The sorting rate was less than 2000 events/s. Sorted cells were collected in 5-mL polystyrene Falcon tubes containing phosphate-buffered saline. Distributions of counts of cells as a function of their light scatter and polyclonal antibody or McB2 antisera) or goat anti-mouse IgM (in the case of the ciliary epithelium of calf eyes by the guanidinium thiocyanate method of Chomczynski and Sacchi (21). RNA samples were fractionated by electrophoresis through a 1% agarose solution containing formaldehyde (11) and transferred onto nitrocellulose filters essentially as described by Towbin et al. (28). The reduction-alkylation of the RNA samples was accomplished by incubation with rhodamine-conjugated goat anti-rabbit or anti-mouse IgG (Fab'), (in the case of 01(2)/1 subunit used in this report have been described already (11) and were provided by Dr. Robert Levenon, Yale University.

Western Blot Analysis—Microsomal fractions from the ciliary epithelium, pars plicata and pars plana regions, were prepared by the method described by Sweadner (24). Protein concentrations were determined by Lowry's method (25). Protein samples were subjected to dithiothreitol and iodosetamide (reduction-alkylation) as described by Charriau et al. (26) before fractionation on sodium dodecyl sulfate-containing 7.5% polyacrylamide gels (30 μg of protein/lane) (27) and were transferred onto nitrocellulose filters essentially as described by Towbin et al. (28). The reduction-alkylation of the microsomal fractions permitted the electrophoretic separation of the α1 isoform from α2 and α3. Polyclonal antibodies to purified dog kidney Na,K-ATPase or rat Na,K-ATPase α subunit (K2) (19) were used to immunodetect the α1 isoform. Monoclonal antibodies McB2 and MCB X3.1 are specific for α2 and α3 isoforms, respectively (15). Polyclonal antisera specific to α2 generated from a TpE-α2 subunit fusion protein was also used (29).

Establishment of a Bovine Ciliary Epithelial Cell Line—A semicon-
Na,K-ATPase Isoforms in the Ocular Ciliary Epithelium

FIG. 2. Flow cytometric analysis of bovine ciliary epithelial cells. SS versus FLS histogram of a preparation of ciliary epithelial cells from the pars plicata region. The selected areas (framed) are comprised of pure populations of PE and NPE cells. Insets represent histograms comparing FLS and SS values between PE and NPE. Pigmented cells had a high SS and low FLS, whereas NPE had low SS and high FLS.

RESULTS

Immunolocalization of the α Subunit Isoforms of Na,K-ATPase in the Bovine Ciliary Epithelium—Previous studies have demonstrated clear ultrastructural differences in distinct regions of the ciliary epithelium—namely, the pars plicata and pars plana (3, 4). Such observations have led to the notion that the former region might be more active in the transport of aqueous humor than the second. In order to determine whether these morphological differences are also reflected in the distribution of the three Na,K-ATPase α isoforms, we have compared the pattern of immunostaining in both regions.

Specific antisera to each of the three α isoforms were applied to semi-thin cryostat sections of 0.5 μm. Figs. 3 and 4 show the pattern with light microscopy of the immunolocalization for the three α isoforms in the pars plicata and pars plana, respectively, of the bovine ciliary epithelium. Polyclonal antibodies to the α (α1) subunit isoform exhibit a distribution

FIG. 3. Immunolocalization of α1, α2, and α3 subunit isoforms of the Na,K-ATPase in 0.5-μm cryostat sections of the pars plicata region of the bovine ciliary epithelium. α1 antisera (polyclonal antibodies to purified canine kidney Na,K-ATPase) stained the basolateral plasma membrane domains of NPE and PE cells (arrows). α2 antisera (McB2) (15) stained only the basolateral plasma membrane of NPE cells, not of the PE cells. α3 antisera (McB-X3.1) (15) also stained the basolateral plasma membrane domain solely of NPE cells, not of PE cells. Specific bound antibodies were visualized with rhodamine-conjugated secondary antibodies. On the right are phase-contrast photographs corresponding to the immunomicrographs on the left.

FIG. 4. Immunolocalization of α1, α2, and α3 subunit isoforms of the Na,K-ATPase in 0.5-μm cryostat sections of the pars plana region of the bovine ciliary epithelium. Antibodies were the same as in Fig. 3. α1 antisera stained the basolateral plasma membrane of the NPE and PE cells; α2 stained the basolateral plasma membrane of NPE cells, but not of the PE. α3 antisera did not stain NPE or PE cells.
throughout the basolateral plasma membranes of NPE and PE cells in both regions (Figs. 3A and 4A). This pattern of staining was identical to the profile obtained with $K_{2}$ antibodies (data not shown). When the monoclonal antibody $McKl$, which lacks cross-reactivity with bovine tissue (15), was tested in rat ciliary processes at the electron microscopic level, we were able to confirm the pattern seen on bovine ciliary epithelium by light microscopy (Fig. 5). Monoclonal antibody $McB2$, which is specific to the $\alpha_2$ isoform, stained the basolateral plasma membrane of NPE cells in the pars plicata and pars plana (Figs. 3C and 4C). However, no detectable signal above background was observed in the pigmented cells with $McB2$, either in the pars plicata or in the pars plana (Figs. 3C and 4C). The staining patterns obtained with $\alpha_1$ and $\alpha_2$ antisera were generally much stronger in the pars plicata region than in the pars plana. Monoclonal antibody $McBX-3.1$, which is specific to the $\alpha_3$ isoform, failed to stain the plasma membrane surface of PE or NPE cells in the pars plana (Fig. 4E). However, the same antibodies stained the basolateral domain of NPE cells in the pars plicata (Fig. 3E).

To verify the failure of $McBX-3.1$ antisera to stain the NPE cells in the pars plana but not of the NPE cells in the pars plicata, both regions were dissected from the same eye and fixed in PLP fixative in the presence or absence of glutaraldehyde (0.08%). Results identical to those shown in Figs. 3E and 4E were obtained. These results raised the questions of (a) whether the labeling epitope (stained by $McBX-3.1$ antibodies in NPE cells of the pars plicata) is not accessible in the NPE cells of the pars plana, and (b) whether the $\alpha_3$ isoform polypeptide was not expressed in the pars plana region of the ciliary epithelium. To address these questions we analyzed the expression of the Na,K-ATPase $\alpha$ isoforms at the mRNA and polypeptide levels in both regions.

**Western Blot Analysis of NPE/PE Cells from Pars Plicata and Pars Planas**—Dissociated ciliary epithelial cells from the pars plicata and pars plana regions were sorted into 100% pure NPE and PE cell populations by flow cytometric analysis as described under “Materials and Methods” (Fig. 6). Microsomal fractions were prepared from both intact tissue and sorted NPE and PE populations and subjected to reduction and alkylation before separation by SDS-polyacrylamide gel electrophoresis (25).

Polyclonal antibodies to the dog kidney Na,K-ATPase $\alpha$ subunit stained a band of 99 kDa in intact tissue and sorted cells (Fig. 6A, lanes 1–3). An identical pattern was obtained for the pars plicata and pars plana, Fig. 6 shows results only from the pars plicata region.

Antisera to $\alpha_3$ (15) reacted with a polypeptide of 105-kDa molecular mass in intact tissue (Fig. 6B, lane 1) and in NPE cells (Fig. 6B, lane 2). No signal was detected by immunoblotting in PE cells (Fig. 6B, lane 3). Identical results were obtained (as shown) for the pars plana.

Due to problems encountered with $McB-X3.1$ on Western blots, we have used a fusion protein antisera to $\alpha_3$ (29) to analyze by immunoblotting the $\alpha_3$ polypeptide expression in intact tissue and sorted cells. In the pars plicata, the fusion protein antisera stained a band of 105-kDa molecular mass in intact tissue (Fig. 6C, lane 1) and NPE cells (lane 2) but not in PE cells (lane 3). An identical pattern was obtained in the pars plana region.

These results indicate that the pars plana and pars plicata regions of the ciliary epithelium expressed three $\alpha$ subunits isoforms of the Na,K-ATPase with a distinct cellular distribution. Although $\alpha_1$ is immunodetected in PE and NPE cells, $\alpha_2$ and $\alpha_3$ isoforms are immunodetected only in NPE cells.

In these studies we have also analyzed the expression of the Na,K-ATPase $\alpha$ isoform polypeptides in a bovine ciliary epithelial cell line established by viral transformation with SV40. All three $\alpha$ isoforms were immunodetected in this cell line in culture (Fig. 6, lane 4, panels A, B, and C). Bovine transformed cells (Fig. 7A) expressed the large T-antigen in 100% of the cells (Fig. 7B) and retained cell polarity only to some degree when grown on Millipore filters (Fig. 7C).

**Northern Blot Analysis**—To analyze expression of Na,K-ATPase $\alpha$ subunit mRNAs in bovine ciliary epithelium and in a bovine ciliary epithelial cell line, cDNA probes specific for rat $\alpha_1$, $\alpha_2$, and $\alpha_3$ subunit isoforms (11) were hybridized to three identical Northern blots, respectively. Each blot con-
transcripts for the three α isoforms were expressed in the ciliary epithelium and in an established cell line. However, clear differences appeared to exist among the three isoforms. Transcripts for the α₁ isoform appeared to be the most abundant, followed by the α₂, with the α₃ transcripts being the least abundant. In several trials, RNA prepared from flow cytometric-sorted cells from the ciliary epithelium was probed on Northern blots with cDNA from the α₁, α₂, and α₃ subunit isoforms. We were unable to detect satisfactory hybridization due to the high level of degraded RNA obtained.

**DISCUSSION**

The regulatory mechanism by which aqueous humor is formed by the ciliary epithelium of the mammalian eye is still largely unknown. The production of aqueous humor depends in part on the specific distribution of pumps, channels, and transporter carriers in the bilayer of cells which comprises the ciliary epithelium. For this reason, the specific localization of the Na,K-ATPase and its α subunit isoforms in these cells is important. We have applied specific antibodies and cDNA probes to the three Na,K-ATPase α subunit isoforms (α₁, α₂, and α₃) to localize their distribution and expression on the ciliary epithelial cells of the bovine eye. We have found that the α₁, α₂, and α₃ subunit isoforms are expressed together in the NPE cells, whereas the α₁ isoform is expressed alone in the PE cells. This finding is consistent with the notion that the NPE ciliary epithelial cells may contribute more than the PE cells to the bulk transport of aqueous humor (6–7). Furthermore, our results on the NPE cells from the pars plicata region of the bovine ciliary epithelium are in agreement with previous immunoblotting studies (5).

**Immunolocalization of the three α isoforms of Na,K-ATPase in the bovine ciliary epithelium indicates that they are exclusively distributed on the basolateral plasma membrane domain of NPE and PE cells.** Previous studies have revealed clear cellular and regional differences in the ultrastructure of the ciliary epithelium of rabbit and primates eyes (3,4). These observations have led to the notion that the pars plicata region, for instance, might be more active than the pars plana in the transport of ions and water into the posterior chamber of the eye. However, information addressing biochemical differences in enzymatic activity of Na,K-ATPase between these two regions of the ciliary epithelium is not yet available. Recent work by Flügel and Lütjen-Drecoll (32) has indicated cytotoxic differences in the distribution of Na,K-ATPase in the pars plicata and pars plana regions of the ciliary epithelium of the rabbit eye. Their results are in contrast with our present findings. In the rabbit ciliary epithelium, Flügel and Lütjen-Drecoll found that in the pars plicata region, Na,K-ATPase was detected in the basolateral and apical plasma membrane of NPE cells without staining the PE cells, whereas in the pars plana region, Na,K-ATPase was found exclusively in the PE cells. One possible explanation of this discrepancy may reside in the specificity and sensitivity of both methods applied to localize the Na,K-ATPase. Flügel and Lütjen-Drecoll used the hidden metal capture method devised by Chayen et al. (33), whereas we have applied isoform-specific antibodies. Another possible explanation is possible species differences in the ciliary epithelium. For example, the bovine ciliary epithelium in contrast to that of the rabbit does not contain iridial ciliary processes.

An interesting finding was that the epitope recognized by McB-X3.1 antibodies (34) in the pars plicata region was not readily accessible to the same antibody in the pars plana. This conclusion was drawn from the observation that a different polyclonal antibody to the α₃ (29) confirmed its presence in...
the NPE cells of the pars plana by immunoblotting analysis. This finding raised two possibilities. The epitope recognized by McB-X3.1 could be occupied by a binding protein in the pars plana. Alternatively, there is a post-translational modification, leading to a different secondary structure of the enzyme. Recent studies have demonstrated that ankyrin links fodrin to the α subunit of Na,K-ATPase in the basolateral plasma membrane of Madin-Darby canine kidney cells (35, 36). This suggests that there are potential proteins that may occupy binding sites (perhaps recognized by McB-X3.1 antibodies) in the α subunit of Na,K-ATPase. Although we do not present here evidence that such protein complexes could interact in the same fashion with the α isoform in the NPE cells of the pars plana, further investigations on the interaction of the membrane-bound Na,K-ATPase and cytoskeleton may provide some information on the binding domains of these proteins. The identification of the specific domain that McB-X3.1 recognizes in NPE cells might be relevant in determining potential regulatory sites modulated by hormones or neurotransmitters.

The expression of only the α isoform in the PE cells is consistent with immunoblotting analysis of membranes from freshly dissociated cells separated by isopycnic sedimentation gradient or by flow cytometry. However, our results cannot rule out the expression of transcripts for α2 and α3, which would indicate a possible transcriptional control, since we were unable to carry out hybridization experiments with intact RNA from sorted cells. Because the PE cells in the ciliary epithelium are continuous with the retinal PE cells, it is interesting to note that both cells express the α isoform. However, the enzyme is distributed differently in the two pigmented cells. The enzyme is localized in the apical membrane of the retinal PE and in the basal membrane of the ciliary PE (57).

A major difference in the expression of α isoform mRNAs by the intact tissue and in the cell line studied was the lack of expression of the 6-kb mRNA species of the α2 isoform. However, the detection of the three α isoform polypeptides with specific isoform antibodies by immunoblotting suggested that the three polypeptides are also expressed in the cell line. These results suggest that the established bovine cell line may originally have been derived from the NPE cell layer, although the possibility of a dedifferentiation in culture cannot be excluded.

Acknowledgments—We thank Dr. Kathleen Sweadner for supplying K2, McK1, McB2, and McB-X3.1 antibodies and Andrew Shyjan for α2 fusion protein antisera. We thank Dr. Robert Post for providing purified canine kidney Na,K-ATPase and Dr. Robert Levenson for cDNA probes. We thank Gill Chatter for expert technical assistance.

REFERENCES

Cellular distribution and differential gene expression of the three alpha subunit isoforms of the Na,K-ATPase in the ocular ciliary epithelium.
S Ghosh, A C Freitag, P Martin-Vasallo and M Coca-Prados


Access the most updated version of this article at http://www.jbc.org/content/265/5/2935

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/265/5/2935.full.html#ref-list-1