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EVIDENCE THAT THE α AND α(+) ISOFORMS OF THE CATALYTIC SUBUNIT OF (Na^+,K^+)-ATPase RESIDE IN DISTINCT CILIARY EPITHELIAL CELLS OF THE MAMMALIAN EYE

Miguel Coca-Prados and Laura G. López-Briones

Yale University School of Medicine, Department of Ophthalmology and Visual Science, New Haven, Connecticut, 06510

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SUMMARY: Polyclonal antibodies against the canine kidney (Na^+,K^+)-ATPase were used to examine the localization and distribution of this protein in intact ciliary processes (CP) from bovine eyes by indirect immunofluorescence. The basolateral surface of non-pigmented (NPE) and pigmented (PE) ciliary epithelial cells was found to be stained specifically for the (Na^+,K^+)-ATPase. Immunoblot analysis of intact CP, separated PE and NPE cells by density gradients and cultured ciliary epithelial cells, revealed two forms of the catalytic subunit of the (Na^+,K^+)-ATPase: the α and α(+). The α(+) form was enriched in NPE cells while α was in PE cells.

The production of aqueous humor is an important physiological process that occurs in the anterior segment of the mammalian eye. It is here where the ciliary epithelium (CE), consisting of a bilayer of epithelial cells, plays a key role in the active transport of ions, proteins and water from the blood vessels into the posterior chamber of the eye (1). An important question relates to the transport properties of these epithelial cells and how a bilayer of cells apposing each other by their apical membranes, coordinates the transport of ions and fluid to form aqueous humor. Electrophysiological evidence indicates that one of the major transport enzymes, (Na^+,K^+)-dependent ATPase (2), that mediates the active transport of $3Na^+$ out of and $2K^+$ into cells, is present in the CE (3). Previous histochemical studies suggest that the (Na^+,K^+)-ATPase in CE is preferentially localized in the basement membrane of non-pigmented (NPE) cells (4,5). Recent studies (6) have shown there is ~2-fold higher (Na^+,K^+)-ATPase activity in dissociated NPE as compared to pigmented (PE) cells.

In recent years accumulated evidence has demonstrated two biochemically distinct isozymes of the catalytic subunit of the (Na^+,K^+)-ATPase, namely the α and α+, in neuronal and non-neuronal cells (7-9). For example, different cell types in the retina have been shown to have either α or α(+), or both isoforms (10). Moreover the retinal PE layer, that contains only the α

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form is embryologically related to the PE of the ciliary epithelium. During the development of the CE the sensory layers of the retina, which comprises about seven different neural cell types (11) and contains both α and α(+) forms (10), become one single layer, the so-called NPE.

In this paper, we explored whether NPE and PE cells contain (Na^+,K^+)-ATPase and whether the two isoforms of the catalytic subunit described in retina cells (7) are also expressed together in the two cell types of the CE, or separately. Using polyclonal antibodies raised against the catalytic subunit of the canine kidney (Na^+,K^+)-ATPase (12) we demonstrated: 1) by indirect immunofluorescence the localization of the (Na^+,K^+)-ATPase in the basolateral membrane domains of both NPE and PE in the ciliary epithelium of the bovine eye, 2) by immunoblot analysis that this antiserum recognizes the α and α(+) subunits of the (Na^+,K^+)-ATPase and 3) these two isoforms are expressed separately in the ciliary epithelial cells; the α(+) preferentially in NPE cells and the α form in PE cells.

MATERIALS AND METHODS

Source of tissue and cell separation - Bovine eyes and bovine brain were obtained from a local slaughterhouse. The ciliary processes (CP) from the eyes of 2 to 5 day old calves were dissected within 2 hr of enucleation as previously described (13). Separation of bovine NPE and PE cells by density gradients was carried out essentially as described (14).

Immunochemistry - Freshly dissected bovine CP were kept in 2.3M sucrose at 4°C overnight before quick freezing in liquid nitrogen. Frozen sections of 0.5 μm were cut using an LKB ultramicrocryotome. Sections were mounted on slides coated with gelatin and used immediately for indirect immunofluorescence. Sections were fixed for 20 min at 4°C with 3% formaldehyde in phosphate-buffered saline (PBS), then treated for 20 min with 0.1M glycine, washed once with PBS and incubated at 37°C with either anti-laminin antibodies (15), kindly provided by Dr. J. Madri, Department of Pathology, Yale University, in a 1:20 dilution or with anti-canine kidney (Na^+,K^+)-ATPase antibodies (12) in 1:200 dilution in PBS containing 0.2% BSA. Second antibodies consisted of fluorescein conjugated affinity purified goat anti-rabbit IgG (Cooper Biomedical, Inc., Malvern, PA). Indirect immunofluorescence of cryostat sections were examined in a Zeiss Universal microscope.

Primary cultures of NPE and PE cells - Primary cultures of bovine NPE cells were prepared in serum-free medium as previously described (13). Primary cultures of bovine PE cells were prepared in serum-containing medium as described (14).

Membrane preparations - Crude plasma membrane preparations of brain, dissected ciliary processes, dissociated NPE and PE, and cultured NPE and PE cells were carried out as described by Sweadner (16). Protein was measured by the method of Lowry *et al.* (17).

Electrophoresis and Immunoblotting - The α and α(+) forms of the (Na^+,K^+)-ATPase from ciliary epithelial cells and brain were resolved on 5% polyacrylamide, one-dimensional slab gels, 25 cm long, according to the method of Laemmli (18).

Immunoblot analysis was carried out essentially as described by Towbin *et al.* (19), with some modifications. Briefly, the electrophoretic transfer of proteins from SDS gels to nitrocellulose was carried out for 18 hours at 18 volts at 4°C. After the transfer, the nitrocellulose was blocked in Blotto (5% w/v nonfat dry milk in PBS and 0.01% Antifoam A) (20) for 1 hour. Fresh Blotto solution containing, at 1:400, anti-canine kidney (Na^+,K^+)-ATPase polyclonal antibodies (12), was incubated with the nitrocellulose filter in "Seal-a-Meal" bags overnight at 4°C with constant rocking. The nitrocellulose filter was then washed 3 times for 5 min each in TBST

(10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20). The filter was then transferred to Blotto containing NBT (nitro blue tetrazolium) with anti-rabbit IgG alkaline phosphatase conjugate (Promega Biotec, Madison, WI) at 1:7,500 dilution and incubated with rocking for 1 h at room temperature. The nitrocellulose was then washed 3 times for 5 min each with TBST and allowed to dry on filter paper at room temperature. Alkaline phosphatase bound to the filter was detected by a color reaction as described by the manufacturer (Promega Biotec, Madison, WI). Protein molecular weight markers, indicated in the legend figure 2, were ß-galactosidase M_r=116,000 and phosphorylase ß M_r=97,400, from Sigma Chemical Co, St. Louis Mo. Immunoblot were scanned using a Shimadzu CS-910 densitometer (Scientific Instruments, Inc.), and the peak area corresponding to each band was integrated by a Chromatopac C-RIA computer (Scientific Instruments, Inc.).

RESULTS

To determine the cell surface polarity of NPE and PE cells in intact CP we utilized rabbit anti-laminin (15) as a probe to visualize the staining of the basement membrane of CE cells. Frozen sections (0.5 µm) of bovine CP were analyzed by indirect immunofluorescence and a narrow zone corresponding to the basal lamina of NPE and PE staining positively for laminin was observed (Fig 1, A-B). This result confirmed previous morphological observations of the cell surface polarity of NPE-PE in vivo (21), with the NPE basement membrane abutting the aqueous humor and the PE apposing the stroma. Furthermore, this result indicates that laminin is not present on the apical cell surface of NPE and PE cells, which are facing each other.

Using rabbit polyclonal antibodies to canine kidney (Na^+,K^+)-ATPase (12) to probe frozen preparations of CP, we observed immunofluorescence along the entire basolateral membrane of NPE and PE cells (Fig 1, C-D); staining on the apical surface of both cell types was absent or comparable to the level observed when preimmune serum was used (not shown). Although we did not quantitate the immunofluorescence signal for (Na^+,K^+)-ATPase fluorescence was routinely greater in the basolateral domain of PE as compared to NPE cells (compare Fig 1,D). We do not know at the present whether this difference in immunofluorescence signal in PE versus NPE reflects differences in structure or number of copies of (Na^+,K^+)-ATPase in the CE cells.

To determine whether the polyclonal antibodies against the canine kidney (Na^+,K^+)-ATPase used in this work recognized both isoforms $\alpha(+)$ and α of the catalytic subunit of brain (Na^+,K^+)-ATPase, we probed a brain microsomal fraction with (Na^+,K^+)-ATPase antibodies. Two bands (Fig 2A, lane 1) of aproximately 100 and 97 kilodaltons (kDa), corresponding to the $\alpha(+)$ and α isoforms of the (Na^+,K^+)-ATPase were detected in the brain (7,9). When similar immunoblot analysis was carried out using microsomes prepared from bovine CP we also detected these two bands (Fig. 2A, lane 5). The M_r of the $\alpha(+)$ subunit of bovine CP was slightly smaller (about 99kDa) than the Mr of the $\alpha(+)$ subunit from brain (about 100kDa). This small, but

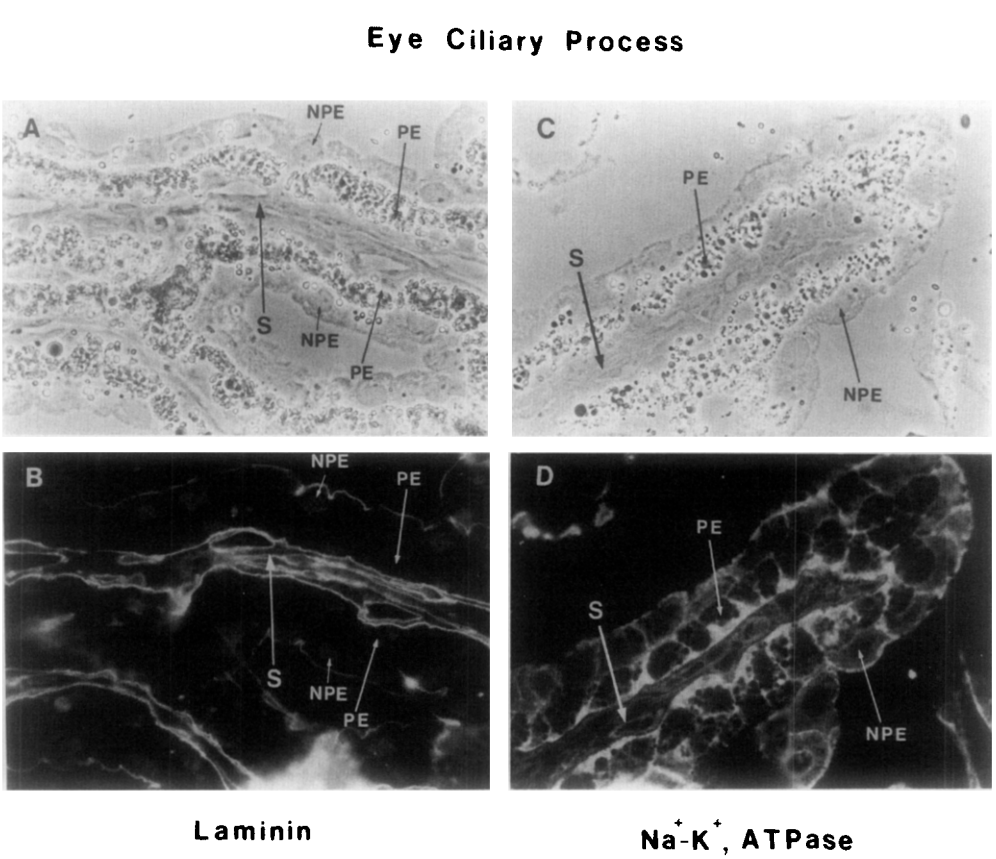

Fig.1. Immunolocalization of laminin and (Na^+,K^+)-ATPase in ciliary processes. Indirect immunofluorescence on cryostat sections (0.5 μm thick) of ciliary processes dissected from bovine eyes showing the cellular polarity of non-pigmented (NPE) and pigmented (PE) ciliary epithelial cells. Immunofluorescence was carried out using rabbit antibodies to laminin (A-B), or using polyclonal antiserum to canine kidney (Na^+,K^+)-ATPase (C-D) as described in the text. A and C are phase contrast micrographs of B and D respectively. The PE layer is distinguished from the NPE layer by the presence of pigmented granules of melanin in the cytoplasm of the former cells. The stroma (S) containing blood vessels is in contact with PE layer. Laminin is distributed in the basement membranes of NPE and PE cell layers (B), which determines the cellular polarity of these cells. The walls of CP blood vessels or stroma (S) are also positive to laminin. There was no detectable signal of laminin in the apical surface of NPE or PE, which are facing each other. The (Na^+,K^+)-ATPase was found to be distributed over the basolateral domain of NPE and PE cells (see arrows in D), with a greater immunofluorescent signal in PE than in NPE. Panel D was overexposed in order to reveal the cells. (x225).

reproducibly measured, difference between α(+) subunit from brain and CP could be distinguished when longer gels (25 cm in length) were run. The M_r of the α subunit from CP was about 97 kDa and thus not different in electrophoretic mobility to the same subunit from brain. When rabbit non-immune serum was used none of the bands seen with the immune serum were detected by immunoblot analysis (not shown).

Next, we looked at the distribution of α subunits in dissociated ciliary epithelial cells. NPE and PE cells were separated on isopycnic density gradients of metrizamide as described previously (14). Fractions with a density of 1.01-1.05 g cm^{-3} were enriched in NPE cells with a

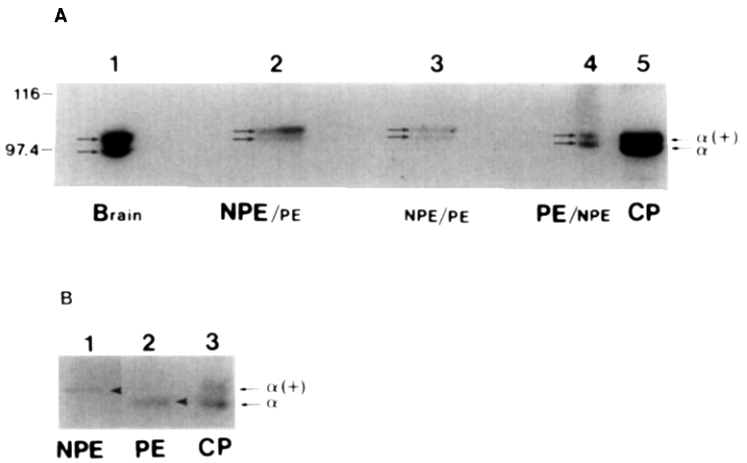

Fig. 2. Identification of α(+) and α isoforms of the (Na^+,K^+)-ATPase in brain and ciliary epithelial cells by immunoblotting. Panel A Western blot analysis of brain microsomes, non-pigmented and pigmented cells and intact ciliary processes. Metrizamide density gradient fractions containing NPE and PE cells were isolated from 30-60% metrizamide gradients, from which microsomes were prepared. Lane 2 represents a fraction close to the top (density 1.01-1.05 g cm^{-3}) of the gradient enriched in NPE cells (about 90% NPE and 10% PE); lane 3 is a fraction (1.06-1.10 g cm^{-3}) where about 55% of the cells were NPE and the rest PE, and lane 4, a fraction (1.18-1.20 g cm^{-3}) where 80% of the cells were PE and about 20% NPE. All samples were solubilized, resolved on a 5% acrylamide SDS gel and tranferred to nitrocellulose paper. The transferred proteins were reacted with rabbit antibodies specific for (Na^+,K^+)-ATPase, and bound antibodies were detected with alkaline phosphatase-coupled anti-rabbit antibodies and substrate. Lane 1: microsomes from brain and lane 5: microsomes from intact CP. Arrows indicate the electrophoretic positions of the α(+) and α subunits of (Na^+,K^+)-ATPase. The ratio between the integrated areas of the scanned α(+) and α bands in the immunoblot were: lane 1, Brain 2.09; lane 2, NPE 3.03; lane 3, NPE/PE 1.57; lane 4, PE/NPE 0.80; and lane 5, CP 1.55. The position and size (in kDa) of the protein molecular weight markers are shown on the left. Panel B: Western blot analysis of microsomes isolated from dissociated bovine CE cells probed with (Na^+,K^+)-ATPase. Conditions were as described above. Primary cultures of bovine NPE and PE cells were prepared as described (12,13). Cells ($2x10^6$) were harvested and a crude microsome fraction prepared as described (7). Lane 1, cultured NPE; lane 2, cultured PE, and lane 3 intact CP. Arrow heads and arrows indicate the position of the α(+) and α isoforms. In panels A and B only the regions containing the α(+) and α isoforms are shown.

10% contamination by PE cells. Fractions with a density 1.18-1.20 g cm^{-3} were enriched in PE cells with a 20% contamination by NPE cells. Fractions with a density between 1.06-1.10 g cm^{-3} contained equal mixtures of NPE and PE cells. Three fractions were selected from the gradient, microsomal membranes were prepared from each and resolved using 5% SDS-PAGE. Proteins were transferred to a nitrocelullose filter and probed with antibodies to the canine kidney (Na^+,K^+)-ATPase. As shown in Fig 2A, (lanes 1 to 4) two bands of M_r 97 kDa and 99 kDa were detected in all three cell fractions containing NPE and PE cells, each with different ratios of α(+) to α isoforms. Both bands have similar electrophoretic mobilities to the α and α(+) derived from intact CP (Fig 2A, lane 5). When immunoblots were quantitated by scanning densitometry the ratio of α(+) to α was determined to be ~2-3, in the NPE-enriched fraction (lane 2) and 0.8 in the PE-

enriched fraction (lane 4). To further determine whether α(+) and α subunits were expressed in both or in separate cells we prepared primary cultures of pure NPE and PE cells as previously described (13,14) and carried out immunoblot analysis. As shown in Fig 2B, while NPE cells in culture expressed α(+) subunit, cultured PE cells expressed only the α subunit.

DISCUSSION

We present evidence that the ciliary epithelium of bovine eye contains (Na^+,K^+)-ATPase distributed along the basolateral domain of NPE and PE cells. This finding, together with the specific localization of laminin on the basement membrane surface of both types of ciliary epithelial cells, may provide a unique way to determine the polarity of the ciliary epithelial cells in vivo. Furthermore, this observation indicates that during the development of the mammalian eye the distribution of (Na^+,K^+)-ATPase, which in the retinal PE is exclusively present on the apical domain (22), is relocated to the basolateral domain of the PE in CE cells. Our data shows that (Na^+,K^+)-ATPase is present on both types of CE cell. This result appears to contradict the notion that (Na^+,K^+)-ATPase transporting activity is exclusively present on NPE cells (5,6). By analogy with the studies carried out by McGrail and Sweadner (1986) on the inmunological distribution of α and α(+) in the retina, one would predict that the PE cells from the CE, which are continuation of the retinal PE, will contain only the α form, while the NPE cells, which are embryologically related to the sensory layers of the retina, will contain both α and α(+) isoforms. Our immunoblot analysis indicated that the α(+) subunit of the (Na^+,K^+)-ATPase is enriched in dissociated NPE cells with minor amounts of the α subunit detectable. In contrast, isolated PE cells are enriched with α. However, the level of α(+) in dissociated PE cells (80-85% pure) could be attributed to the presence of NPE contamination. Nevertheless, it was interesting to observe that in culture each cell type of the CE expressed only one or the other isoform; NPE α(+), and PE α. At the present we cannot rule out the possibility that these cells in culture might express both subunits below the level of detection of the procedures used in this study.

CE cells from different species have been showed to contain a number of adrenergic (α_1, α_2, and ß) and non-adrenergic (VIP, muscarinic-cholinergic, and serotonin) receptors (23). These cells are, therefore, potential targets of a number of hormones and neurotransmitters released under control of the sympathetic system in the proximity of the ciliary epithelium, from the blood or from the posterior chamber side. So it is conceivable that the two α subunits of (Na^+,K^+)-ATPase in the ciliary epithelium might be under differential hormonal regulation as has been

suggested by Lytton *et al.* (9) in rat adipocytes. The availability of different cell clones of NPE and PE from different species (24,25) might facilitate the testing of this hypothesis.

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