Membrane-Associated CA Activity in the Eye of the CA II–Deficient Mouse

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Purpose. Membrane-associated carbonic anhydrase (CA) activity is probably of great importance for transepithelial transport of ions and fluid. Histochemical studies have indicated its presence in the eye, but such histochemical data are difficult to evaluate because of interference from cytoplasmic CA isozymes, of which CA II is predominant. CA II–deficient mice offered the possibility to study the localization of membrane-associated CA activity, without influence from CA II.

Methods. The localization of CA in the eyes of CA II–deficient mice and of normal mice was studied by the cobalt-phosphate histochemical method.

Results. In both types of mice, intense histochemical CA activity was associated with the apical and basolateral membranes of the pigmented and nonpigmented ciliary epithelium, of the corneal endothelium, and of the pigmented epithelium of the retina. It also was localized at the cell borders of the Müller cells and of the lens epithelium and fibers. There also was CA activity in the endothelium of the capillaries of the choroid and retina but not in that of the larger vessels.

Conclusions. Membrane-associated CA activity is found in many ocular cells known to transport fluid and ions. Inhibition of the CA activity of the basolateral membranes of the ciliary nonpigmented epithelium probably explains the reduction of aqueous humor flow seen after the administration of CA inhibitors. Invest Ophthalmol Vis Sci 1994;35:2577–2584.

In mammalian tissues carbonic anhydrase (CA) (carbonate hydrolyase: EC 4.1.1.1) has been shown to exist as seven isozymes, four cytoplasmic designed as CA I, CA II, CA III, and CA VII, one secretory, CA VI, one membrane-bound, CA IV, and one mitochondrial form, CA V. These isozymes have broad structural and evolutionary features in common and all catalyze the interconversion of CO₂ and HCO₃⁻, although at different rates. They are found in many cells, either as the single isozyme or together with the other forms. They are thought to provide the cells with H⁺ and HCO₃⁻ ions for rapid intracellular buffering or for the exchange with other ions, thereby promoting transepithelial movement of ions and fluid.

A systematic biochemical study of the presence of CA in extracts of various ocular tissues from several species, including the rat, was first reported by Bakker in 1941, later followed by similar studies as reviewed by Gloster and Perkins. Histochemical studies of CA activity in the eye was first reported from the rat and mouse by Korhonen and Korhonen, and later from other species, including humans, as surveyed by Lütjen-Drecoll and Lönnherholm. On the whole, these biochemical and histochemical studies show that the distribution of CA activity in the eyes of primates and other mammals is rather similar. Thus, the cytoplasm and the lateral and basal membranes of the corneal endothelium, of the pigmented (PE) and nonpigmented (NPE) ciliary epithelium, and of the retinal pigmented epithelium (RPE) have been found to exhibit CA activity. Such activity has also been found in the apical membranes of NPE and PE and in the cytoplasm and cell borders of the Müller cells. CA activ-
ity has been detected also in corneal epithelial cells and in the capillaries, supplying the deepest layer of the choroid and inner retina, as well as in the stroma underlying the ciliary epithelium.

As to the types of CA isozymes found in the eye, CA II from the bovine lens has been purified to homogeneity and shown to be identical to the erythrocyte CA II. Immunologic and inhibition kinetic studies have shown that the cytoplasmic CA activity in the ciliary epithelium originates from CA II, and there is immunologic evidence for the presence of CA II also in the corneal endothelium, and in the retinal Müller cells and pigment epithelium. Taken together, the current data indicate that CA II is the predominant cytoplasmic CA isozyme in ocular tissues, although there is some evidence that the human corneal endothelium and lens epithelium, and the rabbit corneal epithelium also contain CA I, in addition to CA II.

The membrane-associated CA IV from human kidney and rat lung has been purified and characterized in detail. It was shown to be kinetically similar to the high-activity CA II (with respect to hydration of CO₂ and dehydration of H₂CO₃), but with a 10 to 15 times less sensitivity toward inhibition by sulfonamide inhibitors. By use of selective inhibitors as physiologic tools, CA IV has been found to be more important than CA II for the reabsorption of bicarbonate by the renal proximal tubules. In view of the presence of membrane-associated CA activity in several ocular tissues, known to transport H⁺ and HCO₃⁻ ions, it would seem important to know its precise localizations in these tissues. However, the histochemical findings as cited above, indicating CA activity associated with cell membranes are difficult to evaluate because the resolution of the method does not allow the differentiation between a precipitate formed through the activity of a cytoplasmatic enzyme close to or adhered to the inside of the membrane, and a precipitate formed from the activity of a membrane-bound enzyme. This is also true if EM visualization of the precipitate is used. The recently introduced animal model of CA II deficiency offers a possibility to localize the membrane-bound enzyme in the eye without influence from the cytoplasmic enzyme CA II. With the histochemical technique and CA II-deficient mice, we have therefore reinvestigated the distribution of membrane-associated CA activity in the mouse eye.

**METHODS**

**Animals**

Methods for securing animal tissue were humane and complied with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Three CA II-deficient female mice offspring from intercrossing and backcrossing heterozygotes for the Car² allele, that were homozygous for the Car² mutation (CAD) were used. They lacked the CA II protein in their tissues as tested by immunodiffusion. These mice are smaller than their siblings and appear to have an acidification defect in the distal tubules. As controls, three heterozygote litter mates were used. The animals were anesthetized with pentobarbital sodium and perfusion fixed either with 5% paraformaldehyde (immunohistochemistry) or with Karnovsky's fixative (histochemistry) through the left ventricle of the heart. The whole animal was stored in the fixative at +4°C for 3 days before being processed for histochemical analysis.

**Preparation of Tissue for Microscopy**

After fixation the whole eye was dehydrated through gradedethanols and embedded in the water-soluble resin Historesin for histochemistry as described by Ridderstrale or embedded in paraffin for immunohistochemical staining.

**Histochemical Demonstration of CA Activity**

The cobalt-phosphate histochemical method of Hansson was used in the modified form described by Ridderstrale. From each animal 2-μm-thick sections were cut from different levels of the eyes. The incubation medium contained 3.5 mM cobalt sulfate and incubation times used were 3 and 6 minutes. Throughout the work, the staining procedure was checked by incubation of sections in the presence of 10 μM acetazolamide, a specific inhibitor of CA II and CA IV. Some sections were counterstained with azure blue.

Photomicrographs of sections of CA II-deficient and normal mice were taken under identical conditions using a Nikon Microphot-FXA. All micrographs presented are taken from sections incubated 6 minutes.

**Immunohistochemical Demonstration of CA I and CA II**

Polyclonal antisera against CA I and CA II were raised in New Zealand white rabbits against the rat erythrocyte isozyme CA I and CA II, using standard immunologic procedures. The erythrocyte enzymes were purified to homogeneity as described by Wistrand and Wahlstrand. The specificity of the antiserum for CA I and CA II was determined by immunodiffusion. CA II was found to cross-react with mouse erythrocyte CA II.

Sections 3 to 5 μm thick were put onto gelatin-coated glass slides, deparaffinized and rehydrated through xylol and graded ethanol. The sections were exposed to specific or nonspecific (control) antiserum.
The sites of CA were visualized by the avidin-biotin-peroxidase technique, using a Vectastain kit (Vector Laboratories, Burlingam, CA). A solution of 3-amino-9-ethylcarbazole was used in the final step to visualize the reaction. The sections were mounted under coverslips in glycerin-gelatin. Control experiments included staining after blocking of endogenous peroxidase by exposure of sections to 0.3% H₂O₂. In other control experiments endogenous biotin was blocked, using a blocking kit from the manufacturer.

RESULTS

CA II-Deficient Mice

There were no visual abnormalities of the eyes of the CA II-deficient and control animals, as checked under a dissection microscope.

Immunohistochemistry. The immunostaining indicated lack of CA I and CA II in all tissues of the eye.

Histochemistry. The cytoplasm was unstained in all cells of the eye. The staining was completely abolished by incubation in 10 μM acetazolamide.

Cornea and Lens: Intense staining associated with the basolateral and apical membranes was seen in the corneal endothelium (Fig. 1), in the lens epithelium and in certain equatorial fibers close to the lens epithelium (Fig. 2).

Ciliary Processes: Both NPE and PE were intensely stained along the basolateral membranes, and weakly so at the apical membranes (Fig. 3A). After inhibition by acetazolamide, the staining disappeared and allowed the dissociation of the brownish melanin pigment of the PE cells from the darker histochemical precipitate (Fig. 3B). No staining was found in the capillaries underlying the epithelium.

Retina: Intense staining was seen in the outer limiting membrane and in the inner and outer plexiform layers (Figs. 4, 5A). The nuclei and perikaryons of the cells in the inner and outer nuclear layers were unstained, except for some nuclei in the outer nuclear layer. The cell bodies of these layers were surrounded by thin processes belonging to the Müller cells with intensely stained cell membranes (Fig. 5A). Such processes of the Müller cell close to the inner limiting membrane were also stained (Fig. 5A).

The outer limiting membrane, which is composed of junctional complexes between photoreceptors and...
Müller cells, showed strong staining. Also the microvilli of the Müller cells, surrounding the inner segments of the photoreceptors, appeared to be intensely stained. The nuclei and the cytoplasm of the Müller cells, located in the inner nuclear layer, were unstained (Fig. 5B).

The inner and outer plexiform layers are composed of synapses between the different types of neuronal cells surrounded by processes of the Müller cells. It is likely that the cell membranes of the Müller cells contain CA also at this level. This made it difficult to discern if some of the staining resulted from other cells, such as amacrine, horizontal, or ganglion cells. In any case, the use of CA II–deficient animals made it particularly easy to dissociate the cytoplasmic staining from the membrane staining in the Müller cell. No staining was found in the photoreceptors, nor in the perikaryon of the ganglion cells.

The RPE cells showed intense staining along the basal and apical membranes and weaker at the lateral membranes (Fig. 6A). Again the inhibition by acetazolamide made it possible to dissociate the localization of the melanin pigment and the histochemical precipitate (Fig. 6B).

Vascular Endothelium: Heavy staining was found in the endothelium of the capillaries in the retina (Fig. 5A) and also, but weaker, in the choroid (Fig. 6A). No staining was seen, however, in the larger vessels.

Normal Mice

Immunohistochemistry. The immunostaining indicated lack of CA I in all tissues of the eye but indicated the presence of CA II in the cytoplasm of Müller cells of the retina and in the lens epithelium. No certain staining of CA II was seen in the ciliary processes, in the photoreceptors, in the retinal PE cells or in the capillaries.

Histochemistry. Cytoplasmic staining was only seen in the Müller cells of the retina (Fig. 7). No certain staining was seen in the photoreceptors.
FIGURE 5. CA II-deficient mouse: retina. (A) From top: The inner limiting membrane with intense staining at the cell membranes of the foot processes of the Müller cells. Stained cell membranes of Müller cells are also present in the inner plexiform (IP), inner nuclear (IN), and outer plexiform (OP) layers. Possible staining of cell membranes of other cells (such as amacrine, horizontal, and ganglion cells) is difficult to discern from that of the Müller cells. At the bottom appear intensely stained microvilli detached from RPE. Capillary endothelium is stained (arrows). (Original magnification ×900.) (B) From top: Strongly stained processes of Müller cells surround unstained cell bodies in the outer nuclear layer (ON). The external limiting membrane (arrow) and the microvilli of the Müller cells extending between the unstained photoreceptors are also heavily stained. (Original magnification ×900.)

The plasma membranes of cells in cornea, lens, ciliary processes, retina and vascular endothelium, were stained like the corresponding cells of the CA II-deficient mice. The staining was completely abolished by incubation in 10 μM acetazolamide.

DISCUSSION

Normal Mice

The distribution of CA was similar in all eyes and there was accordance between the histochemical and immunohistochemical findings in the cytoplasm, except for the lens epithelium where the histochemical technique indicated lack of cytoplasmic CA activity, despite the presence of immunologic CA II in these cells. Neither technique could detect any CA staining in the NPE or PE of the ciliary body, despite such activity has been reported for several species. However, the presence of immunologic cytoplasmic staining, has been shown to vary among species and even among mouse species and strains. Thus, in the C57BL/6J mouse, the retinal membranes were stained like the corresponding cells of the CA II-deficient mouse. The staining was completely abolished by incubation in 10 μM acetazolamide.
FIGURE 7. Normal mouse: retina. In addition to membrane-staining of Müller cells as described for the CA II-deficient mouse (Fig. 5), cytosolic staining is seen in Müller cells and their nuclei (arrows). Capillaries are stained (arrowheads). (Original magnification X900.)

ina has been reported to have CA II in the amacrine, horizontal and Müller cells, whereas in our control mice it was only seen in the Müller cells.

Ridderstråle has previously discussed possible causes for lack of histochemical cytoplasmic staining and has emphasized that absence of cytoplasmic enzyme must not be interpreted as lack of such enzyme in vivo. The rather long time of fixation (see "Methods" section) could be one reason for lack of cytoplasmic activity. On the other hand, CA activity associated with the membranes seems to be more resistant to such inactivation.

CA II-Deficient Mice

There was no immunologic evidence of CA II in the eye or other tissues, which agrees with previous biochemical data showing that the CA II protein is lacking in homogenized tissues of these mice. The cytoplasm of the various cells in the eyes also lacked histochemical CA activity, indicating that CA II is the only cytoplasmic CA in the eye that is present in physiologically important concentrations. Therefore, the localization of the membrane-associated CA activity could be easily evaluated in these mice. It was found to compare well with the findings in the control mice, and in the eyes of other species, as discussed in the introduction. This membrane CA activity should correspond with the biochemical findings of CA activity in washed particulate fractions of homogenized bovine lenses, and of isolated bovine PE and human NPE. Recently it was shown that rabbit ciliary epithelium has CA activity originating from an isozyme, biochemically similar to CA IV. However, in the human eye, a recent immunohistochemical study, indicated lack of CA IV in the ciliary epithelium, RPE and corneal endothelium.

Evidently the antibodies used in this study did not recognize the type of membrane-associated CA-isoenzyme found in these tissues.

We propose that the membrane-associated CA activity in the basolateral and apical membranes of ciliary NPE and PE is important for the secretion of HCO₃⁻ into the aqueous humor and of H⁺ ions into the blood, much like what has been proven to be the case for reabsorption of HCO₃⁻ in the renal proximal tubules. Whether this is due to dehydration of H₂CO₃ at the cell exterior, thereby avoiding steep transmembrane pH gradients (and a pH disequilibrium as in the proximal renal tubules) or to the hydration of CO₂, thereby supplying H⁺ and HCO₃⁻ ions for the transmembrane ion exchange mechanisms, is not known. The location of the active site of the enzyme at the cell exterior would favor the dehydration mechanism. Such a location has been made probable in a recent study in which the administration of membrane-impermeable CA-inhibitors on the aqueous humor side of isolated ciliary processes induced a lowering of the transepithelial potential (PD). Such inhibitors will not permeate into the cell to inhibit cytoplasmic enzymes, but will only inhibit a membrane-bound enzyme, with its active site facing the cell exterior. When these inhibitors were added to the blood side no effect on PD was seen. These findings in isolated epithelia therefore imply that the IOP lowering effect of CA inhibitors is due to the inhibition of CA in the basolateral membranes of the ciliary NPE. It is therefore surprising that CA II-deficient humans having normal eyes and IOP, have been reported not to respond with a lowering of IOP after clinical doses of acetazolamide.

The function of the membrane-associated CA in the corneal endothelium, secreting HCO₃⁻ into the aqueous humor, and in the lens epithelium, found to depend on HCO₃⁻ and CO₂ for the translenticular ion transport, should be similar to that in the ciliary epithelium.

It was recently reported that administration of membrane-impermeable inhibitors to the aqueous humor side of the corneal epithelium reduced the transcorneal CO₂ flux, indicating a role for a membrane-bound CA for corneal CO₂ transport.

In the retina, the function of membrane-associated CA in the Müller cells is probably similar to that attributed to this isozyme in the glial cells of the brain, ie, to maintain an optimal electrolyte environment, including HCO₃⁻ ions, for the neuronal activity. It was recently shown that inhibition of membrane-associated CA activity of the glial cells, causes a lowering of the pH-buffering capacity of the brain extracellular fluid. In the inner retina the boundary of the extracellular fluid is formed by the Müller cells, the photore-
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captors and the RPE. The rods acidify the extracellular space in the dark and lactic acid is produced in the outer nuclear layer which generates CO$_2$ by titration of bicarbonate. This metabolic activity creates pH gradients in the extracellular space. The function of the membrane-associated CA of Müller cells, RPE, and retinal capillary endothelium could therefore be to regulate the extracellular pH gradients and maintain a steady concentration of HCO$_3^-$ outside the photoreceptors, an ion shown to be critical for the intracellular pH, and thus for phototransduction.

Key Words
bicarbonate secretion, carbonic anhydrase, histochemistry, immunohistochemistry, cell membranes, ocular tissues, glaucoma, macular edema.

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