Carbonic anhydrase distribution in the rabbit eye by light and electron microscopy

E. Lütjen-Drecoll and G. Lönnérholm

The distribution of carbonic anhydrase (CA) in the rabbit eye was studied by light and electron microscopy according to the histochemical method of Hansson. In the cornea, CA staining was found in the cytoplasm of the endothelium. The filtering tissue in the chamber angle did not stain. The pigmented epithelium of the iris and the non-pigmented epithelium of the ciliary body showed intense staining distinctly located at lateral and apical cell membranes, without clearcut regional differences. In the Müller cells of the retina cytoplasmatic staining was found. The dilatator muscle of the iris, the pigmented epithelium of the ciliary body, and the pigment epithelium of the retina showed similar and intense staining: cytoplasmatic, mitochondrial, and at the cell membranes. The similarity of CA staining in these areas suggests similarity in function, possibly as transport processes important for the nutrition of the overlying cells. However, the role of CA in the various locations remains speculative, except in the corneal endothelium and the prelenticular ciliary processes, where the enzyme is evidently concerned with transport of salt and water. (INVEST OPHTHALMOL VIS SCI 21:782-797, 1981.)

Key words: carbonic anhydrase, histochemistry, rabbit eye

CARBONIC ANHYDRASE (CA) inhibitors have been used in the treatment of glaucoma since 1954, but the cellular localization of the enzyme inhibited by the drugs in the different tissues of the eye has not been studied comprehensively. Previous studies of the ciliary epithelium have revealed marked differences between the shapes and organelle content of the epithelial cells in different regions of the ciliary body. Such differences can hardly exist without corresponding differences in function. It was therefore of special interest to study CA in the different regions of the secretory part of the eye. The opportunity was taken also to observe other ocular tissues.

Material and methods

Light microscopy. Five albino rabbits were killed by intravenous injection of KCl. The eyes were quickly removed, divided, and immersed in 2.5% glutaraldehyde buffered to pH 7.4 by 0.1M phosphate buffer for 4 hr. The eyes from two rabbits were immediately frozen without any fixation by immersion in isopentane cooled by liquid N2. One animal was anesthetized by intravenous injection of a 25% urethane solution, 6 ml/kg, and was then perfused in vivo with 0.9% NaCl followed by fixative, as described above. Some fixed specimens were frozen and stored at —70° C. Others were embedded in the water-soluble resin JB-4 (Polysciences Inc., Warrington, Pa.).4 Frozen tissues, fixed and unfixed, were sectioned in a cryostat at —20° C and were stained for CA activity...
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Fig. 1A. Schematic drawing of the anterior segment of a rabbit eye, demonstrating the different regions investigated. A, Cornea; B, filtering tissue; C, iris; D, ciliary body; I, ciliary iridic processes; 2, prelenticular pars plicata; 3, postlenticular pars plicata; 4, pars plana; E, retina; Re, ciliary recess.

according to the method of Hansson. The resin-embedded tissues were sectioned and stained as described by Riddlerstråle. Fixed sections were stained free-floating, and unfixed sections were supported on Millipore filters. The incubation medium always contained 11.7 mM KH2PO4. Frozen tissues were incubated for 1 to 12 min with 1.75 mM CoSO4 in the medium. Resin-embedded tissues were incubated for 1 to 8 min with 3.5 mM CoSO4. Some sections were counterstained with hematoxylin-eosin.

Electron microscopy. Four albino-rabbits were killed by intravenous injection of an overdose of urethane. The eyes were quickly removed, divided, and fixed in 2.5% glutaraldehyde buffered to pH 7.4 for 2 hr. After being rinsed in buffered sucrose, 20 μm thick sections were cut in a cryostat at —20° C, and the sections were stained for CA as described above. For optimal orientation the stained sections were put on carbon-coated coverslips and embedded in Epon in situ. Ultrathin sections were then cut parallel to the original sectioning direction. Because the stain deposits are located only at the upper (surface) side of the sections, the first ultrathin sections had to be collected to choose the region of optimal staining.

Specificity test. Throughout the work the specificity of the staining procedure was checked by incubation of sections in the presence of 10 μM of acetazolamide (Diamox; American Cyanamid Co., Pearl River, N.Y.), a specific inhibitor of CA. This concentration of acetazolamide completely abolished visible staining (Fig. 5, E) whereas the presence of 10 μM of the inactive control substance CI 13850, a N3-t-butyl analogue of acetazolamide (American Cyanamid Co.) did not interfere with
Fig. 2. A, Sagittal section through the anterior chamber of a rabbit eye after staining for CA and weak counter staining with H&E. (×245.) The corneal endothelial cells (CE) are intensely stained. Staining is also found in the peripheral extension of the endothelium beyond the cornea proper overlying part of the filtering tissue (arrows). F, Filtering tissue; CS, corneal stroma. (Incubation time 8 min; 3.5 mM CoSO₄ in the medium.) B, Sagittal section through the corneal endothelium (CE). (×8000.) The endothelial cells show cytoplasmic staining more intense at the outer surface facing Descemet's membrane (DM). The staining of the cell membranes is difficult to judge because of the dense cytoplasmic precipitates. (Incubation time 9 min; 1.75 mM CoSO₄ in the medium.)

Fig. 3. A, Section through the posterior surface of the iris. (×245.) The pigmented epithelium (P) and the dilator muscle (D) are the only stained structures. (Incubation time 5 min; 3.5 mM CoSO₄ in the medium.) B, Same area as in A. (×8000.) The pigmented epithelium (P) shows intense staining at the basal and lateral infoldings of the cell membrane as well as at the apical cell border facing the dilator muscle. Some stained mitochondria (M) are visible. The dilator muscle (D) shows some cytoplasmic staining in addition to the staining of the cell membrane. (Incubation time 9 min; 1.75 mM CoSO₄ in the medium.)

Results

Sagittal and frontal sections of the whole eye were studied by the light microscope. Sections for electron microscopy were selected from the different regions indicated in Figs. 1A and 1B.

Cornea. In the cornea, CA staining was limited to the endothelium (Fig. 2, A). Staining endothelium was still found at the periphery in the region between the insertions of the iris pillars. The borderline of the staining endothelium coincided with the edge of Descemet's membrane. The membrane, incidentally, extends beyond the corneal stroma into the region of insertion of the pectinate ligament, where it overlies scleral tissue.

the staining. Sections incubated in a medium without any substrate, i.e., NaHCO₃, remained unstained.
Fig. 3. For legend see facing page.
Fig. 4. A and B. A, Section through an iris process. (×245.) The transition area between dilator muscle (D) and PE of the process as well as between pigmented epithelium (P) of the iris and NPE of the process is indicated by arrows. Both cell layers of the process take the stain. (Incubation time 5 min; 3.5 mM CoSO₄ in the medium.) B, Section through the NPE and PE of an iris process. (×4800.) In the NPE cells the lateral infoldings (arrows) and the apical surface of the cell are stained. The pigmented epithelium (PE) in addition shows some cytoplasmic staining. At the apical border well-stained ciliary channels (arrowheads) are visible. (Incubation time 9 min; 1.75 mM CoSO₄ in the medium.)
Chamber angle tissues. The filtering tissue did not stain for CA (Fig. 2, A).

Iris. The pigmented epithelium and the dilatator muscle were the only tissue components that stained (Fig. 3, A). The pigmented epithelium showed very little if any cytoplasmic staining. Intense staining was found at the basal and lateral infoldings and at the surface facing the dilator muscle (Fig. 3, B). The dilator showed some cytoplasmic staining, but the staining again was mainly at the cell membranes.

Ciliary body. The staining of the ciliary body was limited to the epithelium and some of the vessels.

Region 1—iris processes. The iris processes are the forward extension of the ciliary processes to the flat posterior area of the iris (Fig. 1). The low non-pigmented epithelium (NPE) of the iris processes showed no cytoplasmic staining but showed intense staining of the lateral and apical cell borders (Fig. 4, A to C). The pigmented epithelial cells (PE) showed some cytoplasmic staining, but again the cell membranes showed the most intense staining. Clear mitochondrial staining was also found in the PE cells (Fig. 4C). The characteristic extensions of the PE cells toward the stromal capillaries were rich in stained infoldings (Fig. 4C). The microvilli of the ciliary channels between the two epithelial layers were heavily stained (4, B, arrows).

Region 2—pre-lenticular pars plicata with ciliary recesses. In the ciliary processes of this region the cytoplasm of the NPE did not stain, but there was heavy staining at the lateral infoldings and at the apical surface facing the PE. The basal surface did not stain (Fig. 5, A to C). The staining of the apical cell surface showed some interruptions (Fig. 5, B, arrows) at the level where one would expect tight junctions.

In the PE there was clear-cut cytoplasmatic staining, but the most intense staining was at the basal infoldings. Nuclei stained only in resin-embedded preparations (Fig. 5, B, "arrows").
Fig. 5A. Section through the prelenticular ciliary processes and roof of the ciliary recess. (×245.) The high cylindrical NPE cells of the processes (PR) and the valley (V) between the processes show intense staining of the lateral infoldings (arrows). The PE shows intense cytoplasmic staining as well as staining of the cell membranes and basal infoldings. The NPE cells of the roof (R) of the ciliary recess are flatter and show stained basal and lateral infoldings. The cytoplasmic staining of the PE is less prominent than that in the processes. Some stained erythrocytes trapped in unstained vessels are seen (arrowheads). (Incubation time 5 min; 3.5 mM CoSO₄ in the medium.)
Table I. Previous demonstration of the carbonic anhydrase activity in the rabbit eye

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* Iris and ciliary body were homogenized together.
1 Korhonen and Korhonen (1965) studied rat and mouse eyes, and Leder (1966) studied rat retinas with Häusler’s histochemical method.

5A). Some of the capillaries in the processes showed staining of their endothelial lining. The capillaries stained only after comparatively long incubation times (6 to 8 min), whereas, in contrast, the NPE and PE both stained early during the incubation (1 to 2 min).

The NPE and PE of the valleys between the ciliary processes (Fig. 1B) showed the same staining as that described for the ciliary processes themselves (Fig. 5A).

The NPE cells of the roof of the ciliary recesses (Fig. 1B) are low, but still showed basal as well as lateral infoldings, all of which stained. There was no cytoplasmic staining (Fig. 5, A and C).

The PE of the roof showed varying degrees of cytoplasmic staining, but the strongest stain was at the basal surface. On the whole the cytoplasm stained much less here than in the ciliary process PE. No stained capillaries were found.

Fig. 5, D, shows stained erythrocytes in a capillary adjacent to the ciliary epithelium. Fig. 5, E, demonstrates the complete inhibition of CA staining in the ciliary epithelium after incubation of the section in the presence of 10 μM acetazolamide.

Region 3—post-lenticular pars plicata. The ciliary epithelium of these processes showed the same staining as that described for the ciliary processes of the prelenticular pars plicata (Fig. 6A).

In the valleys between the processes the cells of the NPE are taller than those of the processes, and the lateral infoldings, which stained strongly, do not reach down between the cells as deeply (Figs. 6A and 6B). The PE cytoplasm and especially the basal cell border were less stained than in the processes. The basal infoldings were less impressive. No staining capillaries were found.

Region 4—posterior ends of the processes and pars plana. Here the difference between process and valley epithelium disappeared, and surprisingly all cells stained as on the processes. This was also true for the pars plana. No stained vessels were seen.

Retina. The pigment epithelium stained basally and apically, including the microvilli (Fig. 7, A and B). The cytoplasm lacked stain. No stained choroidal capillaries were found. The retina stained very early during incubation, but the stain was limited to the Müller cells and the inner segments of the visual cells (Fig. 7, A). The cytoplasm of the Müller cells was strongly stained and the cell borders formed no distinct contours. In fixed preparations the nuclei of the Müller cells stained (Fig. 7, A). In the inner segments staining was mainly found within the mitochondria (Fig. 7C).

Discussion

Evidence for the specificity of the histochemical method used here has been ad-
Fig. 5, B and C. For legend see facing page.
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Fig. 5, D and E, D, Two erythrocytes in a capillary adjacent to the ciliary epithelium of the prelenticular pars plicata. (×8000.) The cytoplasm is heavily stained. (Incubation time 9 min; 1.75 mM CoSO₄ in the medium.) E, Section through the NPE and PE of the prelenticular pars plicata after incubation of the section in the presence of 10 μM acetazolamide. (×4000.) CA staining is completely inhibited. (Incubation time 9 min; 1.75 mM CoSO₄ in the medium.)

duced by several investigators. As summarized by Lönnroth, the main lines of evidence are (1) excellent correlation between histochemical and biochemical data in tissues studied by both methods, (2) the activity of purified enzyme preparations as measured biochemically agrees with their ability to produce staining in histochemical model experiments, and (3) the staining is inhibited by low concentrations of acetazolamide and other highly active and specific sulfonamide inhibitors of CA. Mulder has questioned the specificity of the method, but the controversy caused by his criticism has been fully resolved by recent work in our and other laboratories.

In the present work, tissues were prepared in several different ways for the incubation. The findings were essentially independent of the preparation. This fact should exclude artifacts in the distribution of the staining caused by diffusion or inactivation of the enzyme. The only clear-cut exceptions are nuclei, erythrocytes, and corneal endothelium. The nuclei in unfixed tissues were always unstained, whereas fixed tissues sometimes showed stained ones. Only cells with cytoplasmic staining had stained nuclei. Such nuclear staining might be nonspecific, as discussed by Hansson, but the matter has not yet been fully clarified. Erythrocytes tended to be less intensely stained in resin-embedded than in frozen tissues (Figs. 6A and 5).

Fig. 5, B and C, B, Section through the NPE and PE of the prelenticular pars plicata. (×8000.) The staining at the apical cell surface of the NPE cells shows some interruptions (arrows). (Incubation time 9 min; 1.75 mM CoSO₄ in the medium.) C, Sagittal section through NPE cells of the roof of the ciliary recess. (×8000.) Basal and lateral infoldings are stained. (Incubation time 9 min; 1.75 mM CoSO₄ in the medium.)
Fig. 6A. For legend see facing page.
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Previous work on the distribution of CA in the eye is summarized in Table I. Our findings agree with those of previous reports on the rabbit eye. However, a systematic study of the different regions of the various tissues has not been made before.

CA activity was presently demonstrated in all parts of the eye derived from the optic cup, i.e., pigmented epithelium and dilatator muscle of the iris, NPE and PE of the ciliary body, and Müller cells and pigmented epi-

Fig. 6B. Section through the NPE and PE of the valley of the posterior pars plicata. M, Stained mitochondria. (×8000.) (Incubation time 9 min; 1.75 mM CoSO₄ in the medium.)

Fig. 6A. Section through the postlenticular pars plicata. (×245.) In the valleys (V) between the processes (PR) the region of stained lateral infoldings does not penetrate as deeply as in the processes (arrows). The pigmented epithelium shows less cytoplasmic staining, and the stained basal infoldings are less prominent than in the processes. (Incubation time 5 min; 3.5 mM CoSO₄ in the medium.)

times found in resin-embedded tissues, have been discussed by Ridderstråle. The corneal endothelium of unfixed rabbit eyes was stained weakly and irregularly, indicating loss of enzyme by diffusion. Because of the several possibilities for lack of cytoplasmic enzyme, we emphasize that absence of stainable cytoplasmic enzyme must not be interpreted as lack of such enzyme in vivo.

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The pigmented epithelium of the retina. In addition, the corneal endothelium showed enzyme activity.

The function of CA in the ciliary body is generally assumed to be to take part in the formation of aqueous humor. The individual roles played in aqueous secretion by the two epithelial layers of the ciliary epithelium are not understood. On the whole there has been a tendency to ascribe the main role in secretion to the NPE. 33, 34

In the monkey, CA staining of the NPE coincides closely with other morphologic indicators of secretory activity. 35, 36 This clear correlation is not seen in the rabbit. In this
Fig. 7C. Inner segment of the visual cells (×20,000.) The mitochondria (M) are strongly stained. Inset, Mitochondria of the inner segment. (×40,000.) (Incubation time 9 min; 1.75 mM CoSO₄ in the medium.)

species, morphologic indicators of secretory functions (i.e., high content of mitochondria and extensive infoldings of the NPE, loosely arranged stromal layer, fenestrated capillaries) are mainly found in the prelenticular pars plicata of the ciliary body. The NPE cells of this region indeed show intense staining. However, staining was also found in the NPE cells of the postlenticular pars plicata and pars plana, where other indices of secretory activity were less prominent.

It is striking that the pigmented epithelium of the ciliary body and retina and the dilator muscle of the iris showed similar intense staining (cytoplasmic, mitochondrial, and at the cell membrane).

It has been assumed that the pigmented epithelium is not important for standing gradient bulk transport of salt and water. Our findings of high CA activity, however, suggest an active role of the pigmented epithelial cells. The close contact between these cells and the stromal capillaries, especially apparent in the iris processes, further supports the idea of actively transporting pigmented epithelial cells.

Although bulk secretion of aqueous humour cannot be an important function of the pigmented epithelial cells, it is tempting to speculate that they are concerned with the nutrition of the overlying cells. This would then be similar to the role suggested for the Müller cells in the retina, which are thought to modulate the environment of the neurons by transporting various molecules, e.g., removal of by-products such as CO₂.

An unsolved question regarding the enzyme of the corneal endothelium is its function in the peripheral extension of the endothelium beyond the filtering angle. But this is only one of several locations of the enzyme for which no function is evident. In fact, direct experimental evidence for a function of the enzyme in any of the locations demonstrated in this paper is available only for the corneal endothelium and the ciliary processes of the rabbit. Fishbarg and Lim, in a study of the rabbit cornea in vitro, found the rate of pumping of the endothelium reduced by the CA inhibitor, ethoxzolamide. Previously, evidence for a bicarbonate-dependent sodium pump in corneal endothelium had been presented by Hodson. Berggren found that the rate at which rabbit ciliary processes in vitro transport stromal fluid and shrink was inhibited by acetazolamide at pH 7 (but not at pH 7.6). It is not certain that the function of CA is always concerned with bulk transport of
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