Carbonic Anhydrase Isoenzymes CA I and CA II in the Human Eye

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The distribution of carbonic anhydrase was studied in human donor eyes by the cobalt-phosphate histochemical method of Hansson and by immunofluorescence and immunoperoxidase techniques using antisera specific against the human cytoplasmic isoenzymes CA I and CA II. Corneal endothelium displayed specific immunological staining for CA I and CA II. Distinct enzyme activity was observed histochemically in the plasma membranes and cytoplasm of the endothelium. In the ciliary processes immunological evidence for the presence of CA II was found both in pigmented (PE) and in nonpigmented (NPE) epithelium. Activity was observed in the cytoplasm and basolateral membranes of NPE, but only in the basal membranes of PE. In the lens the plasma membranes of both the epithelium and fibers displayed intense activity, whereas cytoplasmic enzyme activity was seen only in the epithelium. There was no activity in the lens capsule. Immunofluorescence studies were difficult because of autofluorescence, but the immunoperoxidase technique indicated the presence of both CA I and CA II in the lens. In the central retina, Müller cells stained for CA II. Histochemically, enzyme activity was seen in the cytoplasm and at the plasma membranes. Activity was also observed in some but not all cones. Electron microscopy revealed this to be located in the cristae and plasma membranes adjacent to the pigment epithelium. Activity was also found in PE. Neurons and rods lacked both immunological staining and activity. Endothelial cells of capillaries in ciliary processes and in the choroid stained for CA I and exhibited histochemical activity, particularly those which faced neighboring epithelial cells containing the enzyme. The isoenzyme CA III, which is resistant to inhibition by sulfonamides, did not appear to be present in these ocular tissues, since the histochemical staining of enzyme activity was completely abolished by 10^{-6} M acetazolamide. Invest Ophthalmol Vis Sci 27:419–428, 1986

Human tissues are known to contain three soluble cytoplasmic isoenzymes of carbonic anhydrase (CA) designated CA I, CA II, and CA III. CA II is a high-activity form (with respect to hydration of CO₂ and dehydration of H₂CO₃) that is found in erythrocytes, where it facilitates CO₂ transport. It is also present in secretory epithelia, where it facilitates electrolyte secretion. CA I is a low-activity isoenzyme which occurs in erythrocytes, in certain epithelia of the jejunum and colon, and in the endothelium of capillaries in the lung, kidney, and gastrointestinal tract. Its function is not known. CA III, whose function is also unknown, is a low-activity, sulfonamide-resistant form, found in red fibers of skeletal muscle. Recently, a new hydrophobic membrane-bound isoenzyme, designated CA IV, has been detected in plasma membranes and microsomes of human renal tubular cells. It appears to be involved in the translocation of hydrogen or bicarbonate ions across plasma membranes.

Carbonic anhydrase activity has been found in human ciliary processes, cornea, iris, and retina. CA II has been demonstrated in ciliary processes by immunological and kinetic techniques, and in the Müller cells in the retina by an immunocytochemical technique. There is an obvious need for further studies on the distribution of the various isoenzymes, so that the role of carbonic anhydrase in the ocular tissues can be elucidated. In the present study we have therefore investigated the distribution of the various isoenzymes in human donor eyes by histochemical and immunocytochemical techniques. The histochemical cobalt-phosphate method of Hansson, a well-documented and specific method for demonstrating carbonic anhydrase activity, was used here in a modified form as described by Riddellström, which permitted studies of semi-thin sections by electron microscopy. Immunolocalization of the enzymes was limited to the light-microscopic level and allowed identification of the sites of the two main soluble cytoplasmic isoenzymes, using

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antibodies against CA I and CA II. This technique also served as a valuable control for the histochemical method.

**Materials and Methods**

Normal human eyes intended for corneal transplantation were enucleated 12–24 hr postmortem. They were obtained from the Department of Ophthalmology of the Uppsala University Hospital. The eyes had been stored in cold physiological saline solution for about 3 hr before fixation. Some eyes were dissected, and the various tissues were taken for fixation. Others were fixed by injection of 0.2 ml of 2.5% glutaraldehyde into the vitreous body, whereupon the whole eye was placed in the fixative.

**Tissue Preparation**

For histochemical studies the tissues were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer at pH 7.4, which gave excellent tissue preservation and good histochemical staining. Thin slices were cut and immersed in the fixative for 4–6 hr. After being rinsed in 0.2 M sucrose in 0.05 M phosphate buffer, the tissue slices were embedded in the water-soluble resin JB 4 (Polysciences Inc.; Warrington, PA) or the Sorvall embedding kit (Sorvall, Inc.; Washington, D.C.), sectioned for light and electron microscopy, and stained for carbonic anhydrase as described.

For immunohistochemical studies the tissues were immersed in different fixatives, namely 4% formaldehyde with 0.1% glutaraldehyde, Bouin’s fluid, or Carnoy’s fluid. Fixation in Bouin’s fluid for 6 hr, followed by dehydration through graded ethanols and embedding in paraffin, was found to yield the best material for immunofluorescent staining, in the case of all tissues. Formaldehyde could not be used because of the occurrence of unspecific fluorescence in the whole tissue. Carnoy’s fluid gave good fluorescence but poor tissue preservation. However, in the lens and cornea, autofluorescence was encountered with all fixatives, and for these tissues the pseudoperoxidase (PAP) technique was applied instead.

**Histochemical Staining**

A slightly modified version 15 of the Hansson method 13,14 was used for demonstrating CA activity. The incubation medium invariably contained 3.5 mM cobalt sulfate and the incubation time was 2 to 8 min, since with longer times unspecific precipitation was produced in the sections. For light microscopy, 1–2 μm thick sections were used. Some sections were counterstained with hematoxylin and eosin. The sections for electron microscopy were about 0.1–0.3 μm thick. Reproducible staining could not be achieved with thinner sections.

At least 50 sections of each eye were examined by light microscopy. Adjacent sections from the same block of tissue showed very similar staining.

The specificity of the staining reaction was checked by incubating sections in the presence of 10 μM acetazolamide (Diamox®, American Cyanamid Company; Pearl River, NY), a specific inhibitor of carbonic anhydrase. 17 At this concentration, acetazolamide completely abolished visible staining, whereas the presence of the inactive control substance C113850,17 an N5-t-butyl-analogue of acetazolamide (American Cyanamid Company), at a concentration of 10 μM, did not interfere with the staining.

CA III is a sulfonamide-resistant form of the enzyme, but CA I, CA II, and CA IV are inhibited by low concentrations of acetazolamide (I50 = 0.2, 0.01 and 0.05 μM, respectively). This inhibitor can therefore be used to distinguish CA III activity from that of other isoenzymes in histochemical sections.

**Immunolocalization**

Sections 3 to 6 μm thick of fixed tissues were put onto gelatin-coated glass slides, deparaffinized and dehydrated through xylol and graded ethanol. Sections 6 μm thick of unfixed tissue were cut in a cryostat at −20°C, put onto slides, and fixed in 99% methanol at 4°C for 2 min. The sections were exposed to specific or nonspecific (control) rabbit antiserum at room temperature (22°C). Antisera against the human carbonic anhydrase isoenzymes CA I and CA II (anti-CA I and anti-CA II) raised in rabbits were purchased from Behring Institut, Marburg-Lahn, GFR. The specificities and affinities of the antisera for their respective isoenzymes were determined in our laboratory by immunodiffusion and radioimmunosorbent techniques. 18 The antisera were diluted in phosphate-buffered saline (PBS; 0.8% NaCl in 0.01 M phosphate buffer at pH 7.2) and tested as serial dilutions from 1:10 to 1:2560. Cross-reactivity between anti-CA I and anti-CA II was sometimes observed, but only when the concentration of the nonspecific antiserum was at least 20 times higher than that of the specific antiserum.

**Immunofluorescence Procedure**

After 30 min of incubation with specific antiserum, the sections were rinsed in PBS for 10 min and then exposed to a 1:10 dilution of goat anti-rabbit immunoglobulin labeled with fluorescein isothiocyanate
Fig. 1. Cornea. Immunofluorescence staining with anti-CA I, 1:100 dilution. The endothelium (arrowhead) shows specific fluorescence. S is unstained stroma (×260).

Fig. 2. Cornea. Immunofluorescence staining with anti-CA II, 1:100 dilution. There is strong staining in the endothelium and also some staining also in the corneal stroma (S) (×260).

Fig. 3. Cornea (same as Figs. 1–2). Immunofluorescence staining with non-specific antiserum, 1:10 dilution (×260).

(Behring Institut; Marburg–Lahn, GFR) for 30 min. After a final wash in PBS for 30 min, followed by rinsing in distilled water, the sections were mounted under glass coverslips in PBS-glycerin (1 part PBS, 9 parts glycerin). No counterstaining was used. The slides were examined with use of incident-light excitation with a filter system of type B 450–490/FT 510/LP 520 (Zeiss, GFR, Carl Zeiss, Inc.; Oberkochen, West Germany).
Immunoperoxidase Procedure

In order to block endogenous peroxidase activity and to reduce the nonspecific background staining, sections were first treated for 15 min with methanolic hydrogen peroxide, followed by treatment with 20% normal swine serum. After 60 min of incubation with specific antiserum, the sections were exposed to a 1:20 dilution of swine anti-rabbit immunoglobulin, and then to a 1:80 diluted peroxidase-antiperoxidase (PAP) complex (Dakopatts A/S, Glostrup, Denmark) for 30 min. The peroxidase activity was visualized by incubation for 15 min in 3-amino-9-ethylcarbazole in 0.02 M/l acetate buffer containing dimethylsulfoxide. After each step the sections were thoroughly washed in PBS. The sections were mounted under glass coverslips in glycerol gelatin and examined by light microscopy.

Results

Cornea

Specific fluorescence for CA I and CA II was found in the endothelium. (Figs. 1–3), but the detailed distribution of these enzymes could not be decided. Histochmically, strong activity was found both in the cytoplasm and all around the plasma membranes of the endothelium. In the epithelium weak fluorescence and histochemical activity was seen in some cells, particularly near the limbus. Streaky fluorescence for CA II was also seen in the stroma (Fig. 2).

Ciliary Processes

Intense staining for CA II was observed primarily in the nonpigmented epithelium (NPE), but there was also...
weak staining in the pigmented epithelium (PE) (Fig. 4). There was no clear difference between the epithelia of the valley and crest of the pars plicata. The ciliary epithelium lacked staining for CA I (Fig. 5). Histochemical activity was seen adjacent to the basolateral membranes and in the cytoplasm of NPE (Fig. 6). The basolateral membranes stained clearly, but there was no or only weak cytoplasmic enzyme activity in PE.

The endothelium of capillaries near PE showed specific fluorescence for CA I (Fig. 5) and strong histochemical staining. Erythrocytes trapped in vessels displayed fluorescent staining for CA I (Fig. 5) and CA II and also heavy histochemical staining.

Lens

Owing to autofluorescence it was difficult to discern specific fluorescence for CA I and CA II, but when the pseudoperoxidase method was used instead, both isoenzymes seemed to be present in the epithelium and the lens fibers. Histochemically, strong activity was seen in the epithelium, particularly at the plasma membranes (Fig. 7). The cytoplasm was only weakly stained. Lens fibers showed high activity along the outer cell membranes. No definite cytoplasmic staining was observed. There was no difference in enzyme activity between the different parts of the lens. The lens capsule did not stain histochemically (Fig. 7).

Retina

Immunocytochemically, CA II was found to be evenly distributed in the Müller cells and possibly also in PE, but not in the neurons (Figs. 8–9). CA I was not found in any part of the retina. With the histochemical method strong activity was seen in the cytoplasm and cell membranes of Müller cells (Fig. 10). PE exhibited enzyme activity in the apical and basal membranes, but there was only weak staining in the cytoplasm (Fig. 10). Histochemically, activity was observed in some cones (Figs. 10–11). Findings with the PAP technique also indicated that CA II was only present in certain cones (Fig. 12). Electron microscopic examination revealed that the activity in these cones (Fig. 11) occurred particularly in the part of the photoreceptor bridging across to the pigmented epithelium. The mitochondriarich part of these cones, as well as intracellular (cristae)
and outer membranes was also intensely stained (Fig. 11). None of the rods exhibited any staining (Fig. 10).

The endothelium of the choroid (Fig. 10) stained for CA I. Histochemically the activity was seen to be adjacent to the plasma membranes.

Discussion

General

The distribution of carbonic anhydrase was similar in all eyes tested, and there was good accordance between the histochemical and immunocytochemical findings. The only exception was the corneal epithelium, where the histochemical staining was variable and always considerably weaker than the immunofluorescence. The reason for this discrepancy is not clear at present.

With the histochemical method, staining for carbonic anhydrase activity was found in the endothelium of the cornea, in the pigmented and nonpigmented epithelium of the ciliary processes, in the epithelium and fibers of the lens, in the Müller cells and certain cones in the retina, and in the endothelium of capillaries of the choroid and ciliary processes. With the immunolocalization techniques CA II was found in the same cells of the cornea, ciliary processes, lens, and retina. Moreover, CA I was observed together with CA
II in the endothelium of the cornea, and as the only isozyme in the endothelium of capillaries of ciliary processes and choroid. CA III was probably not present in any tissue, since all histochemical staining was inhibited by 10 μM acetazolamide (see Methods). It should be noted that the immunological method does not detect the membrane-bound CA IV, which is immunologically different from the cytoplasmic forms. With the histochemical method, on the other hand, all isoenzymes, including the membrane-bound form, are detected.

Cornea

We report here for the first time the presence of both CA I and CA II in the corneal endothelium. Previously these two isoenzymes have been found together in the single human erythrocyte and in the surface epithelial cells of the human colon. The function of CA II in the human corneal endothelium, which electrophysiologically behaves like that of the rabbit cornea, is probably to facilitate fluid and bicarbonate secretion. Thus, it has been shown that inhibition of carbonic anhydrase activity in the rabbit cornea causes a reduction in potential across the epithelium (aqueous negative to stroma) and flux of bicarbonate and fluid. The role of CA I in the cornea as well as in erythrocytes and colon cells needs to be elucidated, however.

The epithelium of the human cornea, however, primarily transports sodium chloride to the tear side, and there would appear to be no need for the enzyme for this transport. Using a sensitive biochemical technique, Silverman and Gerster also detected no carbonic anhydrase activity in the stroma or epithelium of rabbit cornea. Our data were inconclusive in this respect.

Ciliary Processes

The presence of CA II in nonpigmented epithelium of the ciliary processes is in accordance with the well-established theory that carbonic anhydrase plays a role in the transfer of sodium bicarbonate and fluid into the aqueous humor. The levels of CA II in the human ciliary processes have been found to be similar to those in renal proximal tubular cells, which constitute another bicarbonate-transporting epithelium. The presence of CA II in the pigmented epithelium suggests that this epithelium is also involved in electrolyte secretion, the nature of which has yet to be determined.

Lens

Carbonic anhydrase has been demonstrated in the lens of many species by use of biochemical, immu-
nochemical, and histochemical methods. These investigations have shown that the enzyme activity varies greatly between different kinds of animals. Thus, the hundred-fold difference between the activity in the lens of the pig and cattle is striking. Very low activity has been found in the human lens, from which both CA I and CA II have been chromatographically separated (Wistrand, unpublished). It has been suggested that carbonic anhydrase, by its catalysis of the hydration of metabolic CO$_2$ and subsequent production of HCO$_3^-$ in the lens fibers, enhances the loss of CO$_2$ by "facilitated" diffusion. The enzyme in the lens epithelium is probably also involved in translenticular electrolyte transport, since in the human lens this transport has been found to depend on the presence of Cl$^-$ and HCO$_3^-$.

**Retina**

The present results confirm the finding of carbonic anhydrase in the primate retina (see Introduction) and add further details concerning the type and distribution of the enzyme in Müller cells, pigment cells, and cones. In Müller cells, the enzyme is located both in the cytoplasm and in the membranes. The function of CA II in the Müller cells might be the same as that attributed to CA II in the glial cells of the brain, that is, to convert CO$_2$ formed in the neurons to bicarbonate and hydrogen ions for use by the glial cells for exchange reactions to maintain an optimal electrolyte environment around the neurons for their activity.

The pigmented epithelium displayed enzyme activity primarily in the membranes. The epithelium of the frog retina has been shown to transport bicarbonate ions toward the choroidal side (or hydrogen ions toward the apical (retinal) side). This transport is inhibited by 10$^{-4}$ M acetazolamide, suggesting that carbonic anhydrase is involved.

Rods did not stain, as reported previously, but in contrast to the previous finding that all cones showed activity, only certain cones were found to be stained. The differential staining of cones and pigmented epithelial cells seems clear, even though the apical processes of the pigmented epithelium are known to be closely associated with the cones. We have not been able to identify the type of the stained cones, but since they constituted about 10% of the cones in the parafoveal area, we suggest that they are identical to the blue-sensitive ones. The function of carbonic anhydrase in the cones needs investigation. It is known that light stimulation of photoreceptors will cause a decrease in their intracellular pH. The enzyme might therefore have a buffering function in these cells. Moreover, it is known that carbonic anhydrase is involved in the exchange mechanism for chloride and bicarbonate in several tissues, and it has been shown that the electrical response of cones, but not of rods, to light is dependent on the chloride concentration in the medium surrounding the receptor. The role of the enzyme could be to furnish bicarbonate ions for this exchange mechanism. Anyhow, it would be interesting to determine whether the function of the blue-sensitive cones is altered by inhibition of their carbonic anhydrase.

**Capillary Endothelium**

The endothelium of the stromal capillaries of the ciliary processes and choroid showed specific fluorescence for CA I. Carbonic anhydrase activity in the capillary endothelium has been demonstrated in skeletal muscle and in lung tissue. Moreover, in the capillaries of the mucosa of the gastrointestinal tract and of the human kidney the enzyme has been shown to be CA I. There is also functional evidence for the presence in the capillary endothelium of a membrane-bound enzyme with its active site facing the blood. In skeletal muscle and the lung, the enzyme is thought to facilitate the transport of CO$_2$ after dehydration of H$_2$CO$_3$. However, the enzyme in the endothelium of capillaries facing secretory cells might not have the same purpose as that in capillaries of the lung and skeletal muscle. The spatial arrangement, with the enzyme activity facing the secretory cells of pigmented epithelium in the retina and ciliary epithelium, suggests that it is involved in the transport of ions (H$^+$ or bicarbonate) from the epithelium to the blood vessel, or in the reverse direction. Why this should be performed by CA I and not CA II, however, is not clear.

**Membrane-Associated Enzyme Activity**

Carbonic anhydrase activity was found to be associated with the plasma membranes in all tissues. Whether this activity originates from a membrane-bound carbonic anhydrase such as CA IV of the kidneys is not known. Biochemical studies have indicated that most of the activity, more than 99%, derives from soluble CA II of the ciliary epithelium and lens. In these tissues only small amounts of activity were particle-bound after homogenization. This contrasts with the histochemical finding of strong activity in the plasma membranes and relatively weak activity in the cytoplasm of many cells. In a previous histochemical study of the primates, frozen tissues showed stronger cytoplasmic staining than tissues which had been resin-embedded as in the present study.
Carbonic Anhydrase Inhibition

Inhibitors of carbonic anhydrase play an important role in ophthalmology, where they are used to reduce elevated intraocular pressures. Apart from transient myopia and blurred vision, no adverse reactions from the eye have been described in spite of long-term treatment of patients, often for years. This is surprising in view of the presence of carbonic anhydrase in several ocular tissues. Acetazolamide is the inhibitor mostly used, and the reason for its lack of effect on tissues other than ciliary processes might be that its concentration in the aqueous humor, lens, and vitreous is low as a result of active transport out of ocular tissues, as has been found in rabbits. Acetazolamide is also 20 times less active against CA I than against CA II. However, this does not seem to explain the lack of toxicity in the eye since the other available clinical inhibitors are all more lipid-soluble than acetazolamide and therefore attain higher concentrations in the eye. Moreover, they are all equally active against CA I and CA II. One of them, methazolamide, has been used clinically for almost as long a time as acetazolamide and yet does not seem to have a higher toxicity in the eye. Whether inhibition of carbonic anhydrase in cornea, lens, and retina has any functional significance, therefore, remains to be elucidated.

Key words: carbonic anhydrase, cornea, ciliary body, iso-enzymes, lens, retina, glaucoma

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