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Localization of carbonic anhydrase IV in a specific capillary bed of the human eye

(choroid/choriocapillaris/glaucoma)

GREGORY S. HAGEMAN*, XIN LIANG ZHU†, ABDUL WAHEED†, AND WILLIAM S. SLY†

*Bethesda Eye Institute, Department of Ophthalmology, St. Louis University School of Medicine, 3655 Vista Avenue, and †Edward A. Doisy Department of Biochemistry and Molecular Biology, St. Louis University School of Medicine, 1402 South Grand Boulevard, St. Louis, MO 63110

Contributed by William S. Sly, December 20, 1990

ABSTRACT **Carbonic anhydrase (CA) activity plays an important role in controlling aqueous humor production in the eye and in regulating intraocular pressure. Prior studies identified the soluble isozymes CA II and CA I in the human eye and also suggested a distinct membrane-associated CA. We used an antibody to CA IV, the membrane-anchored isozyme from human lung, to study CA IV in eye tissues and to compare its distribution with that of CA II. We found intense immunostaining for CA IV associated with endothelial cells of one specific uveal capillary bed, the choriocapillaris. CA IV was not detected in endothelial cells of the contiguous capillaries of the iris or in endothelial cells of other vessels. Immunoreactivity for CA IV was also intense in epithelial and fiber cells of the lens but was not detectable in the neuroretina, the ciliary process (except for capillaries), and the cornea, all sites where immunostaining with anti-CA II antibody was intense. These studies indicate that the membrane-associated CA in human eye, which was suspected from histochemical studies, is CA IV. Defining the physiological role of this ocular isozyme remains a challenge.**

Seven carbonic anhydrase (CA) isozymes have been identified in mammals (1). They differ in physical and kinetic properties, in susceptibility to inhibitors, in subcellular localization, and in tissue-specific expression (2, 3). Ocular CAs have received much attention because of their importance in glaucoma (4). Normal intraocular pressure is regulated in part by aqueous humor production, which depends on bicarbonate secretion by the ciliary epithelium. CA inhibitors reduce aqueous humor production and lower intraocular pressure (5).

CA activity has been demonstrated in ocular tissues from rabbits, monkeys, and humans (6–14). Immunochemical studies identified CA II as the most widely distributed CA in the eye (15–18). It is especially abundant in epithelial cells of the ciliary process, in Muller cells of the retina, and in a subset of cone photoreceptor cells. CA II is also an important marker in cellular differentiation of ocular tissues (19–23). Offspring of mice (23) and chickens (19) treated with CA inhibitors during pregnancy develop microphthalmia or anophthalmia. The fact that these findings were not seen in humans (24) or mice (25) with CA II deficiency suggests that CA II is not the sole target of CA inhibitors that perturb eye development.

CA I, another soluble isozyme, has been identified in corneal endothelial cells, lenticular cells, and capillary endothelial cells in the stroma of ciliary processes and the choroid (15). CA III, another soluble isozyme, was not detected in the eye (15). A membrane-associated CA was detected by histochemical studies that did not react with antisera to the soluble isozymes. CA IV is a membrane-associated CA in lung and kidney, which was recently purified and characterized (26, 27). In the studies reported herein, we used an antibody generated against purified CA IV from human lung and an antibody to purified CA II to determine whether the membrane-associated enzyme in human eye was CA IV, to delineate its localization, and to compare its distribution to that of CA II.

MATERIALS AND METHODS

Antibody Production and Purification. Antibody to human lung CA IV (27) was produced in New Zealand White rabbits, purified by immunoaffinity chromatography on an Affi-Gel 10–CA IV affinity column, and adsorbed against purified CA I and CA II. Rabbit antibody to recombinant human CA II (28) expressed in *Escherichia coli* was the generous gift of Donna Roth of this laboratory.

Tissue Collection. Six pairs of adult human eyes were obtained from donors, 24–82 years of age, through the American Red Cross Eye Bank (St. Louis) and Mid America Transplant (St. Louis). Tissues were processed within 2 hr following death; no pathology was noted by macroscopic or microscopic examination. One eye from each pair was bisected and fixed by immersion in 4.0% formaldehyde (freshly generated from paraformaldehyde) in 100 mM sodium cacodylate buffer (pH 7.4) for 1–2 hr. Eyes were subsequently rinsed for 60 min in three changes of 100 mM sodium cacodylate buffer (pH 7.4). Ocular tissues, including cornea, iris, lens, ciliary body, pars plana/choroid/sclera, and retina/choroid/sclera, were embedded in acrylamide (Polysciences), mounted in Tissue Tek OCT (Miles Scientific), frozen in liquid nitrogen, and sectioned on a cryostat at −20°C as described (29). Some tissues were embedded in OCT directly, without fixation, frozen in liquid nitrogen, and sectioned at −20°C.

Immunofluorescence Microscopy. Tissue sections were incubated for 15 min in phosphate-buffered saline (PBS) (pH 7.4) containing 1.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, and globulin-free bovine serum albumin at 1.0 mg/ml, followed by a 10-min rinse in PBS containing 1 mM $MgCl_2$ and 1 mM $CaCl_2$. Sections were subsequently exposed to affinity-purified anti-human lung CA IV antibody or to anti-human CA II antibody in PBS for 1–2 hr in a humidified chamber. Sections were then rinsed extensively in PBS and incubated with affinity-purified, fluorescein-conjugated, goat anti-rabbit IgG (Sigma) for 30–60 min in a dark, humidified chamber. Controls included (*i*) incubations with preimmune serum in place of specific primary antibody and (*ii*) incubations in secondary antibody alone. Sections were rerinsed, coverslipped in Immunomount (Shandon, Pittsburgh), and examined by epifluorescence microscopy, using an Olympus Vanox-T light microscope. Images were recorded on Kodak Tri-X film by

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Abbreviations: CA, carbonic anhydrase; RPE, retinal pigmented epithelium (epithelial).

using an exposure index of 800. Photographs were taken and printed at the same exposure to allow direct comparisons of binding intensity.

Ocular Homogenates. Lens, iris, cornea, choroid/retinal pigmented epithelium, and neural retina were dissected from the second eye of each pair, frozen in liquid nitrogen, and stored at −80°C. Subsequently, they were thawed and homogenized using a Bronsonic homogenizer in an equal volume of PBS or double-concentrated sample buffer for SDS/PAGE (30). The protein concentration of each homogenate was determined by using bicinchoninic acid (BCA) reagents (Pierce) and bovine serum albumin as a standard.

Gel Electrophoresis and Immunoblotting. Approximately 30 μg of each tissue extract was subjected to one-dimensional SDS/PAGE by using a 12% acrylamide gel (30). The separated proteins were transferred electrophoretically to Immobilon-P membranes (Millipore) by using an SDS electroblotting system (Millipore) as described (31). Blots were equilibrated in Tris-buffered saline (20 mM Tris·HCl and 150 mM NaCl at pH 8.0) containing 0.05% Tween 20 and 2.0% casein to block nonspecific binding sites. The blots were subsequently exposed to antibodies directed against human lung CA IV (200 ng of IgG per ml) or human CA II (diluted 1:1000) in Tris-buffered saline containing 0.05% Tween 20 for 2 hr at room temperature, as described (27). Antibody-incubated blots were rinsed in Tris-buffered saline containing Tween 20 and subsequently exposed to horseradish peroxidase-conjugated goat anti-rabbit IgG (Sigma). Colored reaction products of bound horseradish peroxidase-conjugated antibody were generated by using H_2O_2 and 4-chloro-1-naphthol. This reaction was terminated by rinsing in distilled water. Controls for the specificity of antibody binding included (*i*) the use of preimmune serum in place of specific antisera and (*ii*) application of the second antibody alone.

RESULTS

Neural Retina/Retinal Pigmented Epithelium (RPE)/Choroid. Anti-human CA II antibody reacts strongly with the inner retina (Fig. 1*A*). Muller cells, glial cells that span the neural retina from the inner limiting membrane to the outer limiting membrane, are labeled intensely. In addition, cone, but not rod, photoreceptor cells are labeled by anti-CA II antibody. Labeling is visible over the cone photoreceptor cell bodies, axons, and the inner and outer segments (Fig. 1*A Inset*). Labeling is especially intense at the inner segment/outer segment interface. Other neuronal cells, as well as the endothelial cells of larger retinal vessels, are not labeled by anti-CA II antibody. Anti-CA II antibody reacts weakly with RPE cells (Fig. 1*A*).

In contrast, anti-CA IV antibody does not label the neural retina or RPE (Fig. 1*B*). However, endothelial cells of the choroidal capillary bed, the choriocapillaris, are intensely and specifically labeled by antibody directed against CA IV (Figs. 1*B* and 2). No other vessels within the choroid (including arterioles and venules) are labeled by this antibody. In addition, no reaction of anti-CA IV antibody is observed with endothelial cells of vessels at sites of attachment to the capillaries of the choriocapillaris (Fig. 2*A*). A discontinuity of endothelial-associated anti-CA IV labeling is always observed at sites where efferent or afferent vessels attach to the choriocapillaris. At higher magnification (Fig. 2 *C* and *D*), labeling with anti-CA IV antibody is observed on both the luminal and adluminal surfaces of the endothelial cells; this is especially apparent in regions where the luminal and adluminal surfaces are clearly separated by endothelial cell nuclei (Fig. 2 *C* and *D*). The endothelial cells of the choriocapillaris reactive with anti-CA IV antibody do not react with anti-CA II antibody (Fig. 1*A*).

Ciliary Body. The distribution of labeling of the ciliary body by anti-CA IV antibody is distinct from that seen with anti-CA II antibody. Anti-CA IV antibody reacts specifically with the choriocapillaris overlying the ciliary epithelium of both the pars plana and pars plicata regions (Fig. 3 *A* and *C*). Endothelial cells of capillaries located within the stroma of ciliary processes are also labeled by anti-CA IV antibody (Fig. 3*C*) but not by anti-CA II antibody (Fig. 3*D*). The ciliary muscle and stroma are not labeled by either antibody.

Although anti-CA IV antibody does not label either the pigmented or nonpigmented epithelial layers of the ciliary body (Fig. 3 *A* and *C*), anti-CA II antibody intensely labels both. This is true in both the pars plana and pars plicata regions (Fig. 3 *B* and *D*). Immunoreactivity is typically more intense in the nonpigmented ciliary epithelial layer as compared to the pigmented ciliary epithelial layer in both the pars plana and pars plicata. The immunoreactivity associated with the nonpigmented epithelial layer is more uniformly distributed in the pars plicata than in the pars plana.

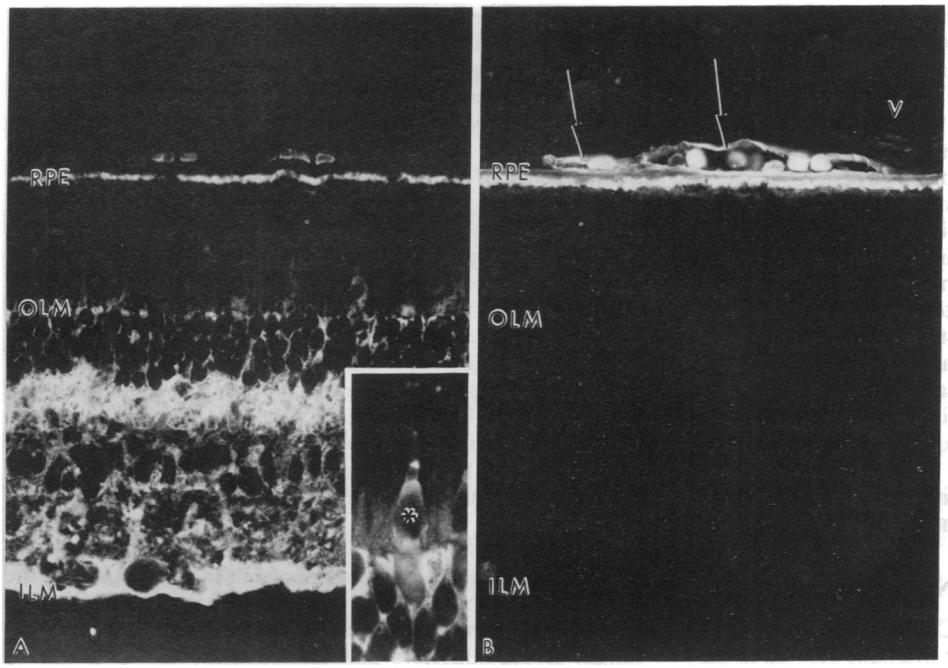

FIG. 1. Fluorescence light micrographs of sections of human retina immunostained with anti-CA II (*A*) and anti-CA IV (*B*) antibodies. Anti-CA II antibodies intensely label the inner neural retina, primarily labeling Muller cells, which extend from the inner limiting membrane (ILM) to the outer limiting membrane (OLM). Some cone photoreceptor cells are also labeled by this antibody (*Inset*). In contrast, anti-CA IV antibodies do not label the retina. However, the anti-CA IV antibody intensely labels the endothelial cells of the choriocapillaris (arrows). Other large vessels (V) present in the choroid are not labeled by this antibody. (×330.)

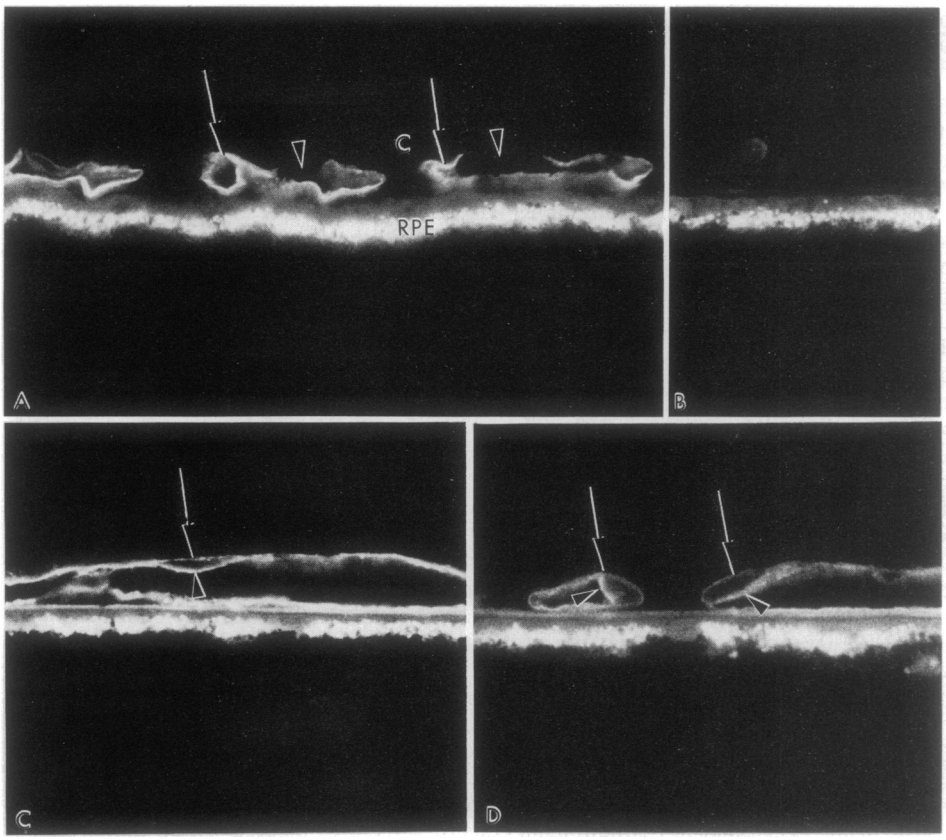

FIG. 2. Fluorescence light micrographs of sections of human RPE and choroid (C) showing specific reaction of the choriocapillaris with anti-CA IV antibody. Specific labeling of the endothelial cells of the choriocapillaris (arrows in *A*), but not the endothelium of larger vessels of the choroidal stroma, is apparent. (*B*) Control sections treated with preimmune serum followed by second antibody do not react. Discontinuity of choriocapillaris-associated anti-CA IV binding is observed at sites where larger efferent and afferent vessels merge with the endothelium of the choriocapillaris (arrowheads in *A*). In addition, anti-CA IV immunoreactivity appears to be associated with both the luminal (arrowheads in *C* and *D*) and adluminal (arrows in *C* and *D*) surfaces of the choriocapillary endothelium. Autofluorescence of the RPE is evident in *A–D*. (*A–C*, ×430; *D*, ×490.)

Iris. Neither anti-CA IV antibody nor anti-CA II antibody binds to any portion of the iris, including the two epithelial layers, stroma, and/or dilator muscles (Fig. 3 *E* and *F*).

Lens. Lens fiber cells are labeled by both anti-CA IV (Fig. 4*A*) and anti-CA II (Fig. 4*B*) antibodies. Labeling by anti-CA II is especially intense in the cortical region of the lens. The distribution of CA IV in the lens epithelial cells is more intense in the equatorial region where dividing lens epithelial cells are elongating into lens cortical cells (not shown). Anti-CA II antibody also binds weakly to lens epithelial cells. Neither antibody binds to any portion of the lens capsule (Fig. 4).

Cornea. Anti-CA IV antibody does not label any region of the cornea (Fig. 5 *A* and *C*). In contrast, anti-CA II reacts intensely with the corneal endothelium (Fig. 5*D*) and sporadically with keratocytes (not shown). No labeling of either antibody to the corneal and conjunctival epithelium is observed.

Trabecular Meshwork, Sclera, and Vitreous Body. Neither anti-CA II nor anti-CA IV antibody labels any portion of the sclera, vitreous body, or trabecular meshwork.

Immunoblotting. Western immunoblotting was carried out on extracts of ocular tissues with antibodies to CA IV and CA II (data not shown). The anti-CA IV antibody reacted with a 35-kDa protein (the molecular mass of native CA IV) in extracts of choroid, lens, and iris. It also reacted with a 16-kDa band, which is near the molecular mass of proteolytic fragments of CA IV in urinary membranes (32). No reactivity with anti-CA IV antibody was seen in extracts of retina or cornea. Anti-CA II antibody identified a 30-kDa band in extracts of choroid, neuroretina, iris, and lens. The immunoblot data agree with the immunohistochemical results except in the iris, where we assume that the CA IV immunoreactivity reflects incomplete dissection and removal of choroid from the iris.

DISCUSSION

The studies presented here identify CA IV in the human eye and define its distribution. The most dramatic finding is the intense and specific immunostaining for CA IV in endothelial cells of the choriocapillaris (15). Immunoreactivity for CA I in endothelial cells of the choriocapillaris has also been reported (15). Presumably CA I is expressed in the cytoplasm of the endothelial cells, whereas CA IV is expressed on the exterior surface of the plasma membrane. An example of such dual localization of different CAs is found in epithelial cells of the proximal tubule of the kidney, which contain both a soluble and a membrane CA (32, 33). The cytosolic isozyme in this case is CA II, and the cell surface isozyme is CA IV, which is expressed on both the apical and the basolateral surface of the epithelial cells.

Although immunoreactivity for CA IV was observed in the highly fenestrated, permeable capillaries associated with the choroid and ciliary process, it was not seen in the highly impermeable capillaries of the iris, a vascular bed with which the choriocapillaris is contiguous. A similar, sharply defined pattern of expression has been observed in the renal tubule, where CA IV is expressed in the brush border of the S1 and S2 segments of the proximal convoluted tubule but is not expressed at all in the brush border membranes of adjacent cells in the S3 segments (34).

The physiological roles of the different CAs in the eye probably vary with cell type and with intracellular localization. CA IV in the choriocapillaris could participate in the transport of ions and/or CO_2 between the vasculature and the RPE by ensuring a rapid equilibrium of the CO_2, HCO_3^-, and H^+ surrounding the capillaries. Its intimate spatial association with the RPE could make this possible. Although it is well documented that there are fluxes of HCO_3^-, CO_2, and H^+ across the retinal pigmented epithelium (35), little is known about the regulation of these ions on the choroidal side of the RPE.

Determining whether CA IV contributes to maintenance of intraocular pressure may be important in understanding the role of CA inhibitors in the treatment of glaucoma. CA II in the ciliary epithelium has been assumed to be the primary target of CA inhibitors that reduce intraocular pressure by

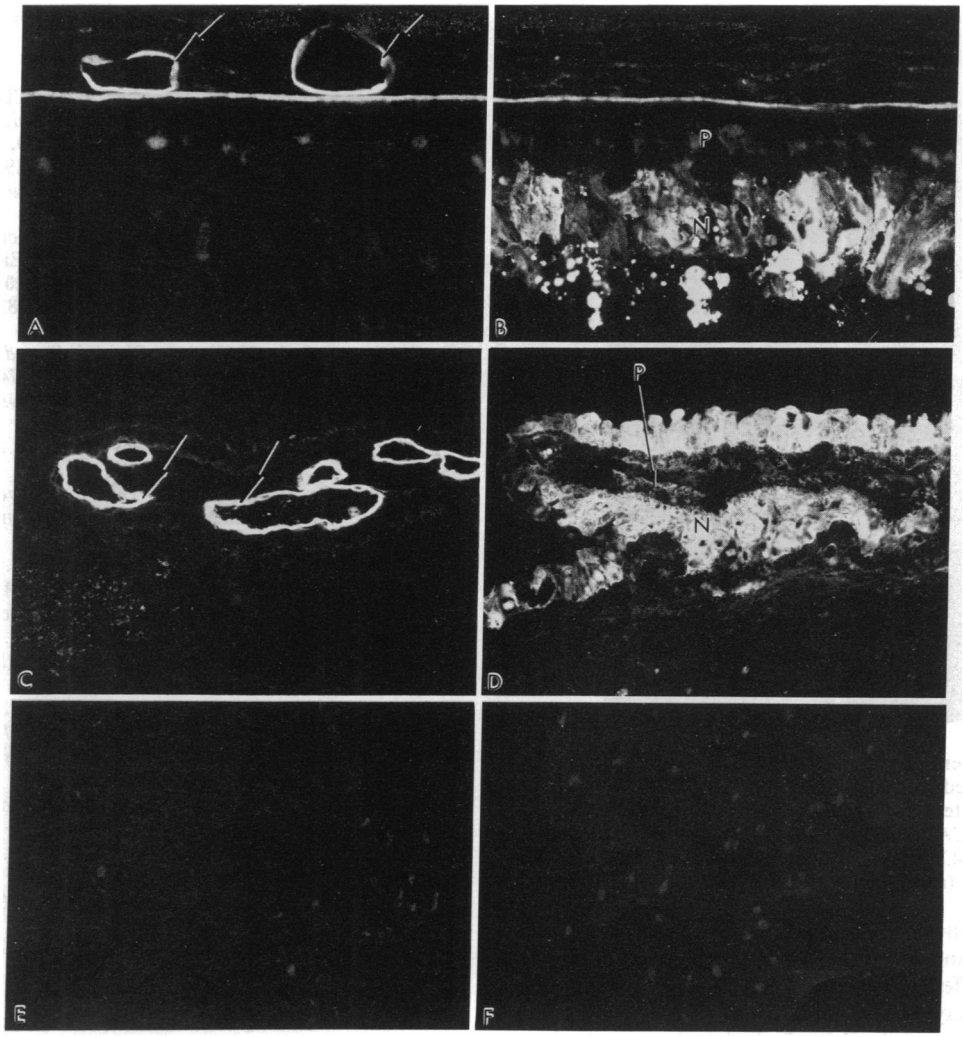

FIG. 3. Fluorescence light micrographs of serial sections of human ciliary epithelium/choroid in the region of the pars plana (*A* and *B*), the ciliary processes in the region of the pars plicata (*C* and *D*), and the iris (*E* and *F*). These sections show the distribution of immunoreactive CA IV (*A*, *C*, and *E*) and CA II (*B*, *D*, and *F*). Anti-CA IV antibody specifically labels the capillary bed overlying the ciliary epithelium of both the pars plana and pars plicata (arrows in *A* and *C*). Anti-CA II antibody, in contrast, labels both the pigmented (P) and nonpigmented (N) epithelial layers of both regions but not the capillary beds within the stroma (*B* and *D*). Neither antibody labels the iris (*E* and *F*). (*A* and *B*, ×270; *C* and *D*, ×180; *E* and *F*, ×230.)

inhibiting aqueous humor production (33). However, CA IV is similar to CA II in sensitivity to sulfonamide inhibitors (27). It will be interesting to determine whether inhibitors routinely used to inhibit CA II in the ciliary processes mediate some of their effects through inhibition of CA IV. If CA IV in the choriocapillaris normally acts in concert with CA II in the ciliary process to maintain a balanced intraocular pressure, it could explain why exposure of fetal rats and chickens to CA inhibitors produces microphthalmia, which is not seen in the isolated, inherited deficiency of CA II (23–25).

This study shows that CA IV immunoreactivity is also associated with the lens. Previous biochemical, immunohis-

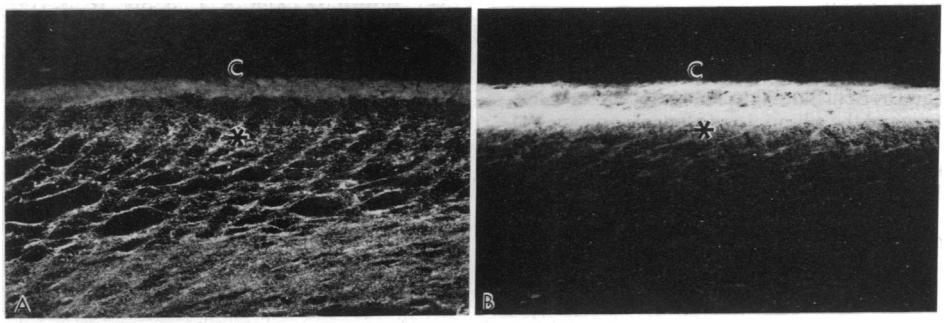

FIG. 4. Fluorescence light micrographs of human lens immunolabeled with anti-CA IV (*A*) and anti-CA II (*B*) antibodies. Both antibodies label the lenticular cells, including both the fiber and epithelial cells (asterisk). No binding is observed with the lens capsule (C). Binding of anti-CA II is more intense in the cortical region of the lens (*B*), whereas binding of anti-CA IV appears evenly distributed throughout the lens. (×200.)

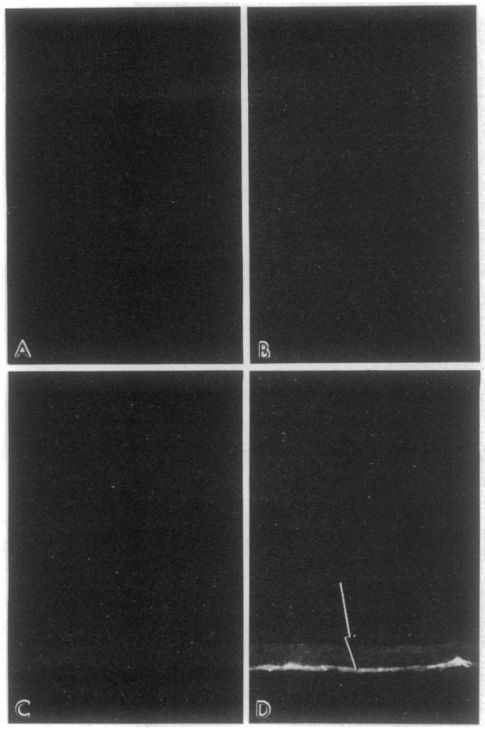

FIG. 5. Fluorescence light micrographs of sections of human cornea immunostained with anti-CA IV (*A* and *C*) and anti-CA II (*B* and *D*) in both the anterior (*A* and *B*) and posterior (*C* and *D*) regions of the cornea. Anti-CA IV immunoreactivity is not observed in the cornea. Intense anti-CA II immunoreactivity is associated with the corneal endothelium (arrow in *D*). (×250.)

tochemical, and histochemical studies have demonstrated the presence of CA in this tissue, though its activity varies significantly in different species. It has been suggested that CAs in the lens may enhance the loss of CO_2 by facilitated diffusion and/or be involved in electrolyte transport, since translenticular electron transport is dependent on the presence of Cl^- and HCO_3^- (36). Clearly, the function of CA IV in the human lens warrants further study.

Clinical observations in CA I- and CA II-deficient patients have been extremely helpful in delineating the respective physiological roles of these two isozymes (24, 37, 38). On the basis of this experience, identification of patients with CA IV deficiency and description of the ocular abnormalities of CA IV-deficient patients seem worthwhile goals. Such observations might disclose the specific functional roles of CA IV in the choriocapillaris and lens of the human eye, analogous to the way in which clinical observations on patients with CA II deficiency defined the roles of CA II in bone resorption and renal acidification (24, 37, 38).

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