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Carbonic anhydrase IV on brain capillary endothelial cells: A marker associated with the blood–brain barrier

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ABSTRACT **Carbonic anhydrase (CA) activity plays an important role in controlling cerebrospinal fluid production and also influences neuroexcitation and susceptibility to seizures. Until recently, CA II was the only CA demonstrated in brain. Its distribution is limited to the epithelial cells of the choroid plexus and to the myelin-forming cells, the oligodendrocytes. In this report, we present immunoblots, using an antibody raised to CA IV from rat lung, that show that CA IV is also present in rat and mouse brain. Results of immunohistochemistry and immunoelectron microscopy on sections from rat and mouse brain are presented that show the distribution of CA IV to be quite distinct from that of CA II. CA IV is expressed on and is limited to the luminal surface of endothelial cells of cerebral capillaries. These results establish CA IV as a cytochemical marker associated with the blood–brain barrier and suggest an important role for CA IV in CO_2 and HCO_3^- homeostasis in brain.**

The carbonic anhydrase (CA) gene family contains seven genes (1, 2), each of which encodes a distinct CA isozyme. These isozymes (CA I–CA VII) differ in kinetic properties, in susceptibility to inhibitors, in subcellular localization, and in tissue-specific expression (3, 4). To date, only CA II has been reported to be intrinsic to nervous tissue (5–7), although small amounts of CA III transcript have been detected in mouse brain (8). The CA II in the central nervous system is limited to the myelin-producing cells, the oligodendrocytes (5–7, 9–11). The presence of CA II gene transcript in mixed primary cultured glial cells is also restricted to oligodendrocytes (12, 13).

CA IV is a membrane-associated CA that has been purified from human kidney (14) and human lung (15). The cDNA for human CA IV was recently cloned and characterized (16). Human CA IV has an apparent molecular mass of 35 kDa, is insensitive to endoglycosidases, and contains no N-linked or O-linked oligosaccharide chains (15). Much of the CA IV in kidney and lung is anchored to membrane by glycosyl phosphatidylinositol linkages (15). Immunolocalization studies on human eye tissues showed that CA IV is highly expressed in a specific capillary bed, the choriocapillaris (17).

Rat CA IV also has been purified from rat lung and found to be a glycosyl phosphatidylinositol-anchored 39-kDa protein containing one N-linked oligosaccharide chain (18). Its distribution was found to be quite widespread. In addition to lung and kidney, where prior immunolocalization studies had shown it to be present on apical and basolateral surfaces of certain segments of the rat nephron (19), it was identified in tissue extracts of many other organs including brain. In the present paper, we report the cellular and ultrastructural localization of CA IV in the central nervous system in the rat and in the CA II-deficient mouse. In both species, its distribution in the central nervous system is limited to the luminal surface of capillary endothelial cells, suggesting an important functional role at the blood–brain barrier.

MATERIALS AND METHODS

Tissue Preparation. Brains from adult Wistar rats and from CA II-deficient (*Car-2*^{0}) mice (7) were fixed by transcardiac perfusion with 4% paraformaldehyde in phosphate-buffered saline (PBS) and then kept in the same fixative for 2–4 hr at 4°C. Eighty-micrometer-thick Vibratome sections were cut and washed thoroughly in PBS before immunostaining.

Immunocytochemistry. To reduce the endogenous peroxidase activity and the nonspecific binding of antibodies, tissue sections were incubated for 10 min with 3% hydrogen peroxide in methanol. After washes in PBS, tissue sections were incubated for 1 hr with 3% (wt/vol) bovine serum albumin (Sigma) and 10% (vol/vol) nonimmune sheep serum in PBS.

Tissue sections were incubated 1–2 hr at room temperature with a 1:500 dilution of the rabbit antiserum to rat lung CA IV, which reacts with CA IV of the same molecular weight in membranes from rat brain (see *Results*). Some sections were incubated with a 1:200 dilution of rabbit antiserum to rat CA II (7). After several washes in PBS, sections were incubated for 1 hr at room temperature with peroxidase-conjugated goat anti-rabbit IgG (Sigma) at 1:100 dilution. The peroxidase activity was developed with 0.02% 3,3′-diaminobenzidine tetrahydrochloride and 0.003% hydrogen peroxide. Some control tissue sections were incubated with rabbit nonimmune serum and processed for immunoperoxidase as mentioned above. Tissue sections were examined with a Leitz orthoplan microscope. For electron microscopy, after examination by light microscopy, selected tissue sections were postfixed with osmium tetroxide, dehydrated in alcohol, and flat-embedded in Spurr resin (TAAB Laboratories, Reading Berks, U.K.) (20). Ultrathin sections were examined with a Philips 420 electron microscope (Philips Electronic Instruments, Mahwah, NJ) without counterstaining.

Antisera Preparations. Antisera to rat lung CA IV and to human and rat CA II were prepared as described (15, 19).

Gel Electrophoresis and Immunoblotting. Brain protein (30 μg) from rat and from normal (+/+) and CA II-deficient mice (*Car-2*^{0}) was subjected to SDS/PAGE followed by immunolabeling with rat lung CA IV or human CA II antiserum as described (19).

Phosphatidylinositol-Specific Phospholipase C Treatment of Brain Tissue. Brain protein (100 μg) from rat was treated with 0.09 enzyme unit of phosphatidylinositol-specific phospholipase C (from ICN) or buffer alone as described (18). Membrane-bound and soluble forms of CA IV were analyzed by SDS/PAGE followed by immunoblotting.

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Abbreviation: CA, carbonic anhydrase.

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RESULTS

Immunoblot Data. Antiserum raised to the 39-kDa CA IV from rat lung reacted strongly with a 39-kDa protein in rat brain (Fig. 1*A*). This 39-kDa protein was reduced to 36 kDa by deglycosylation with peptide *N*-glycosidase F (18). A faint band at 45 kDa was also seen in rat brain (Fig. 1*A*) and occasionally in other tissues, but this appeared to be a nonspecific reaction as it also was seen with nonimmune serum (not shown). CA IV in normal and CA II-deficient mice was slightly smaller than 39 kDa. A small amount of immunoreactive protein was seen in mice brain extracts at 35 kDa, the same molecular mass as that of nonglycosylated murine CA IV. Fig. 1*A* also shows that the antiserum to rat CA II recognized a polypeptide of 29 kDa in the brain of rat and normal mouse that was completely absent from the brain of the CA II-deficient mouse.

Fig. 1*B* shows that the majority of CA IV (<90%) in rat brain was bound to the membrane. However, upon treatment with phosphatidylinositol-specific phospholipase C, >80% of

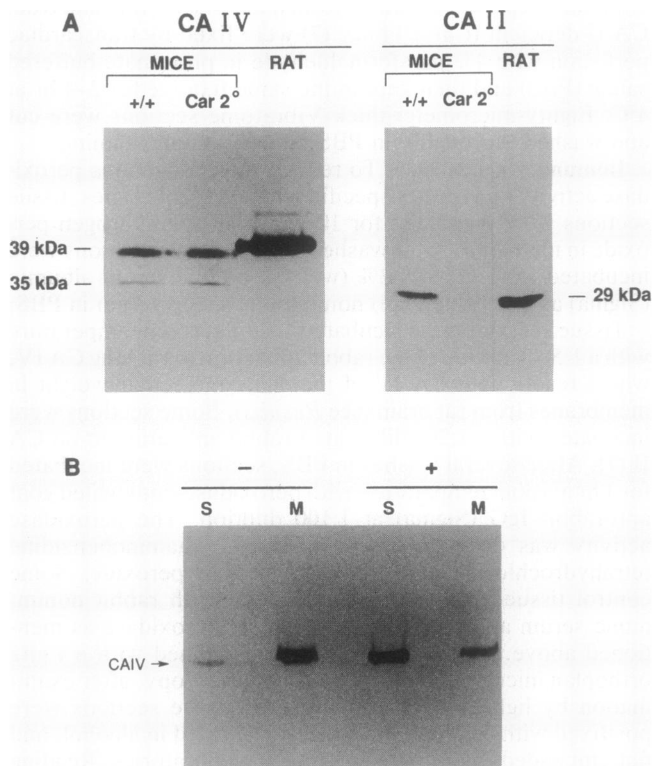

FIG. 1. (*A*) Immunoblot of CA isozymes from mouse and rat brain. Tissue protein (30 μg) from a normal mouse (+/+), a CA II-deficient (*Car-2*^{0}) mouse, and rat (RAT) was subjected to SDS/PAGE followed by immunoblotting with rat lung CA IV antiserum (CA IV) or human CA II antiserum (CA II), as indicated. The apparent molecular masses in kDa of the polypeptides are marked. The CA IV in mouse brain is slightly smaller than 39 kDa. The 35-kDa protein in mouse represents the nonglycosylated form and has the same molecular mass as human CA IV, which contains no carbohydrate (5). The anti-CA IV antiserum reacts with a 39-kDa protein in rat brain—the same mass as that of CA IV from rat lung (18). A small amount of an immunocrossreactive polypeptide at 45 kDa is seen in rat brain and occasionally in other rat tissues that also is seen with nonimmune serum. Antiserum to CA II reacts with a polypeptide of 29 kDa in both normal mouse and rat. No crossreacting polypeptide for CA II was seen in brain from CA II-deficient *Car-2*^{0} mice. (*B*) Rat brain tissue (100 μg) was treated with buffer alone (lanes −) or 0.9 enzyme unit of phosphatidylinositol-specific phospholipase C (lanes +). Soluble (lanes S) or membrane-associated (lanes M) enzyme was recovered by centrifugation and subjected to SDS/PAGE followed by immunoblotting. An arrow indicates the rat brain CA IV standard (CA IV).

the enzyme was solubilized. These results indicate that the CA IV in rat brain is anchored to membranes via a glycosyl phosphatidylinositol linkage, as is CA IV in human lung and kidney (15) and CA IV in other rat tissues (18).

Localization of CA IV at the Light Microscopic Level. In adult rat cerebral cortex (Fig. 2 *A* and *B*), the immunoperoxidase precipitate for CA IV was limited to blood vessels. Neuronal and glial cells were unstained for CA IV. CA IV was also present in blood vessels of CA II-deficient mice (Fig. 2*C*). A control experiment with CA II antiserum (Fig. 2*D*) showed staining exclusively in oligodendrocytes in rat cerebral cortex, while blood vessels and other structures were unstained. Note that the epithelium of the choroid plexus was heavily stained for CA II (Fig. 3*A*), whereas it remained unstained for CA IV (Fig. 3*B*) except for the blood vessels crossing through this epithelium. The rat brain sections incubated with nonimmune rabbit serum showed no specific staining (Fig. 3*C*).

Ultrastructural Localization of CA IV. The immunoperoxidase reaction product denoting the presence of CA IV was limited to capillary endothelial cells (Fig. 4 *A–C*). In general, the luminal surface of endothelial cells was heavily stained for CA IV, whereas the cytoplasm and cytoplasmic organelles were negative for this isozyme. No specific staining could be detected in other cells or structures surrounding the blood vessels (Fig. 4 *A–C*). The specificity of CA IV immunostaining was demonstrated by using a rabbit antiserum to rat CA II to immunostain sections from cerebellar cortex under identical conditions (Fig. 4*D*). No staining for CA II was observed in blood capillary endothelial cells or in astrocytic end feet surrounding blood vessels. The immunoreaction product for CA II was restricted to the cytoplasm and plasma membrane of oligodendrocytes.

DISCUSSION

CA II has been considered to be the principal form of CA present in the central nervous system (5–7, 21, 22). From immunochemical studies, it has been concluded that CA II is restricted to oligodendrocytes and the epithelium of the choroid plexus (5–7, 23, 24). Although most of the CA II is present in soluble forms in newborn rat brain, an increasing fraction appears in the particulate fraction during postnatal development, a change that appears to be correlated with myelination (6). In adult rat and mouse brain, 55% of CA II is myelin-associated, though it can be extracted from myelin by solubilization of the particulate fraction with Triton X-100 (6).

The membrane association of CA IV in rat brain is due to its glycosyl phosphatidylinositol anchor, as evidenced by its release from membranes by treatment with phosphatidylinositol-specific phospholipase C (Fig. 1*B*). Similar evidence for a glycosyl phosphatidylinositol anchor has been reported for CA IV in human lung and kidney membranes (15) and for CA IV in lung membranes of nine additional mammalian species (18). The immunocytochemical studies presented in Figs. 2–4 show that the luminal surfaces of cerebral capillaries are specifically and intensely stained with antibodies to CA IV. Preferential localization of CA IV at the plasma face of the cerebral capillaries is, presumably conferred by the glycosyl phosphatidylinositol anchor, which is thought to provide a general mechanism for targeting membrane proteins to the apical surfaces of polarized cells (25).

Brain capillary endothelial cells are characterized by the presence of tight junctions and by the absence of fenestrations. Thus, the monolayer of endothelial cells could function as a continuous membrane (26, 27). The presence of enzymes such as γ-glutamyltranspeptidase (28–30), angiotensin-converting enzyme (31, 32), and Na^+/K^+-ATPase (33) and other components including factor VIII (34), prostacyclin (35), and receptors (36) give unique properties to these

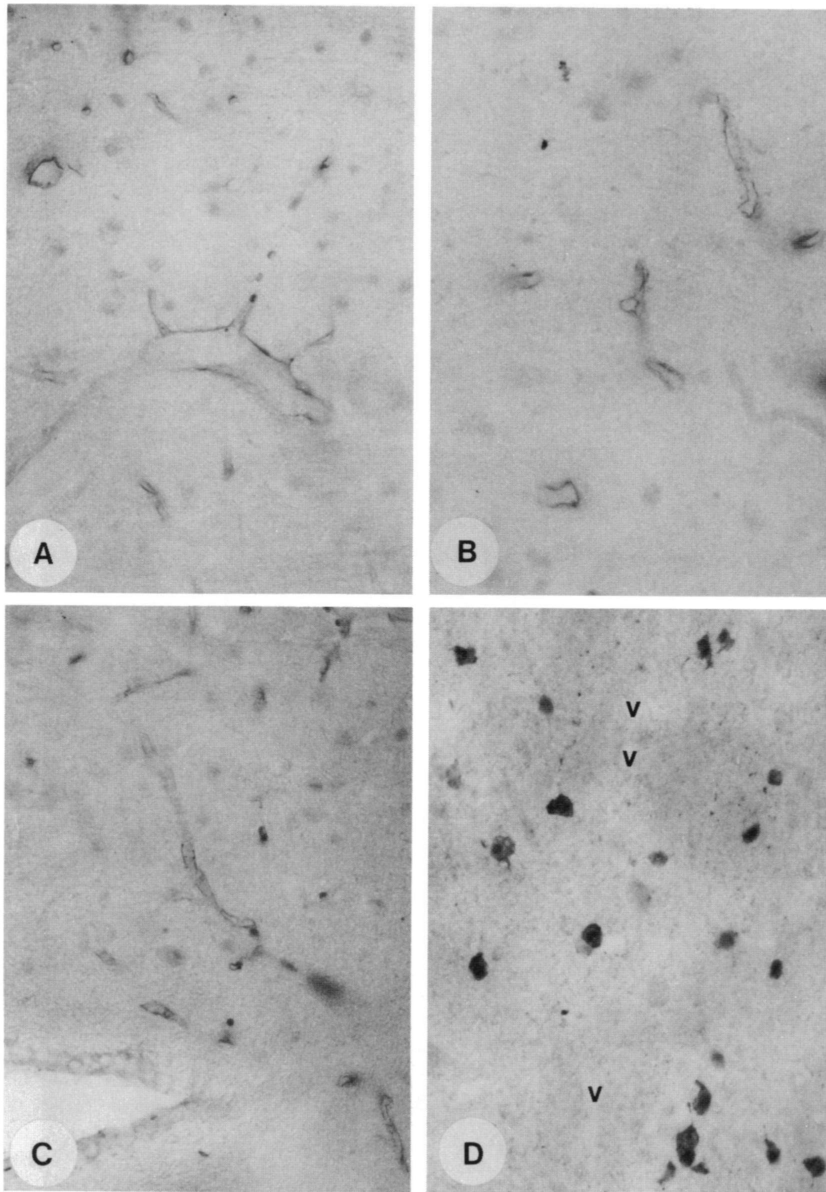

FIG. 2. Immunoperoxidase staining for CA IV (*A–C*) and CA II (*D*). Cerebral cortex from adult rat (*A* and *B*) and from CA II-deficient mouse (*C*) stained with anti-CA IV antiserum. Intense labeling decorates the walls of blood vessels (*A–C*). The control section stained for CA II shows strong labeling of oligodendrocytes in rat cerebral cortex (*D*). Note that blood vessels (V) are negatively stained for CA II. (*A–D*, ×230.)

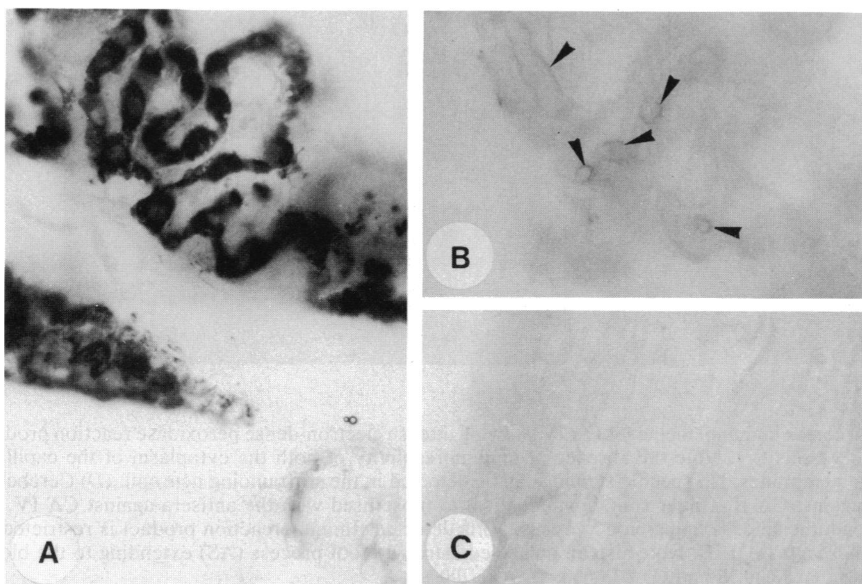

FIG. 3. (*A*) The epithelial cells of choroid plexus in adult rat brain are heavily stained for CA II with immunoperoxidase. (*B*) The epithelial cells of choroid plexus are negative for CA IV, while the nearby blood capillaries are stained (arrowheads). (*C*) Cerebral cortex from adult rat stained with rabbit nonimmune serum and processed for immunoperoxidase. No significant staining is observed. (*A–C*, ×230.)

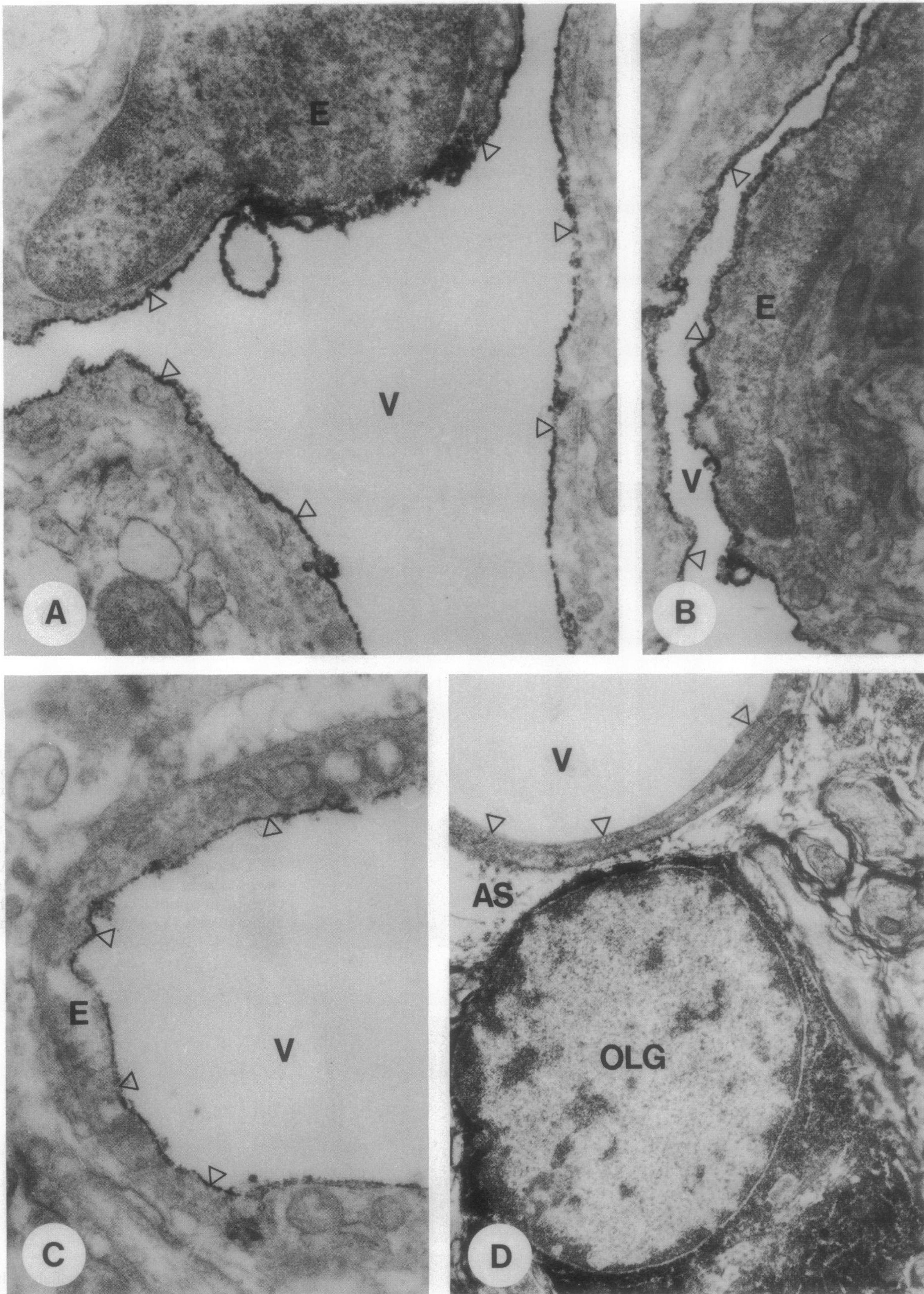

FIG. 4. (*A–C*) Electron micrograph of cerebral cortex immunolabeled for CA IV showing intense electron-dense peroxidase reaction product (arrowheads) lining the luminal surface of blood vessels (V). Note the absence of immunoreactivity of both the cytoplasm of the capillary endothelial cells (E) and their nonluminal plasma membranes. No specific staining can be detected in the surrounding neuropil. (*D*) Cerebellar cortex immunolabeled with antiserum directed against CA II. Under conditions identical to those used with the antisera against CA IV, no staining of the luminal surface (arrowheads) of endothelial cells lining blood vessels (V) is detected. Immunoreaction product is restricted to the cytoplasm and plasma membrane of oligodendrocytes (OLG). Note also an unlabeled astrocyte foot process (AS) extending to the blood vessel. (*A–D*, sections were not counterstained; *A*, ×23,600; *B* and *C*, ×18,000; *D*, ×11,700.)

endothelial cells. CA IV can now be added to this list. Since the vascular surface of the cerebral capillaries represents the major portion of the blood–brain barrier, CA IV can be considered an immunohistochemical marker for the blood–brain barrier.

The strategic localization of CA IV at the luminal face of the cerebral capillaries and its physiological properties as a high-activity isozyme suggest that CA IV may have an important role in CO_2 and HCO_3^- homeostasis in the brain. Other membrane surfaces where CA is present in high concentrations—i.e., on the apical surface of proximal tubule cells in kidney and on the luminal surfaces of endothelium of pulmonary capillaries—are sites where large fluxes of CO_2 and HCO_3^- occur. In lung, even before morphological support for such an enzyme localization and distribution was provided (37, 38), use of blood-free perfused isolated animal lung preparations led physiologists to postulate that a CA was bound to the luminal surface of pulmonary endothelial cells (39–41). Studies on the effect of CA inhibitors using this system suggested a role for the luminal CA in CO_2 transport from lung capillaries, the quantitative importance of which is still debated (42). By analogy, the cerebral capillary CA could be physiologically important for CO_2 transport from brain to plasma. CA-catalyzed hydration of CO_2, which is freely diffusible across the brain capillaries, would form H^+ and HCO_3^- in plasma that are not freely diffusible. This reaction could greatly facilitate CO_2 transport from brain to plasma. Enzyme inhibitors which blocked this CA-catalyzed hydration reaction might produce CO_2 retention in brain.

Before brain CAs were fully characterized, neurological effects of CA inhibitors were attributed to secondary effects of inhibition of erythrocyte CAs (43). More recently, it has been suggested that inhibition of CA II in brain might account for some of the neurological side effects of CA inhibitors (44). However, the fact that CA II is limited to oligodendrocytes and choroid plexus makes it unlikely that inhibition of CA II is the explanation for the peripheral effects of these inhibitors, such as their effects on neuroexcitation and seizure activity. On the other hand, the finding that CA IV is located on the luminal surface of cerebral capillaries and associated with the blood–brain barrier, makes CA IV an attractive candidate to explain the cortical effects of CA inhibitors that influence brain function.

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