

Expression of membrane-associated carbonic anhydrase XIV on neurons and axons in mouse and human brain

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Although long suspected from histochemical evidence for carbonic anhydrase (CA) activity on neurons and observations that CA inhibitors enhance the extracellular alkaline shifts associated with synaptic transmission, an extracellular CA in brain had not been identified. A candidate for this CA was suggested by the recent discovery of membrane CA (CA XIV) whose mRNA is expressed in mouse and human brain and in several other tissues. For immunolocalization of CA XIV in mouse and human brain, we developed two antibodies, one against a secretory form of enzymatically active recombinant mouse CA XIV, and one against a synthetic peptide corresponding to the 24 C-terminal amino acids in the human enzyme. Immunostaining for CA XIV was found on neuronal membranes and axons in both mouse and human brain. The highest expression was seen on large neuronal bodies and axons in the anterolateral part of pons and medulla oblongata. Other CA XIV-positive sites included the hippocampus, corpus callosum, cerebellar white matter and peduncles, pyramidal tract, and choroid plexus. Mouse brain also showed a positive reaction in the molecular layer of the cerebral cortex and granular cellular layer of the cerebellum. These observations make CA XIV a likely candidate for the extracellular CA postulated to have an important role in modulating excitatory synaptic transmission in brain.

Carbonic anhydrases (CAs) are produced in a variety of tissues where they participate in a broad range of physiological processes such as acid-base homeostasis, carbon dioxide and ion transport, respiration, bone resorption, renal acidification, gluconeogenesis, ureagenesis, and formation of cerebrospinal fluid and gastric acid (1–5). The growing α -CA gene family includes 11 enzymatically active members with different structural and catalytic properties. Several CA isozymes are expressed in the central nervous system where each of them has a characteristic distribution pattern. Cytosolic CA II is present in the oligodendrocytes and myelin sheaths (6, 7), and some investigators have also demonstrated positive signal in the astrocytes (8, 9). Membrane-bound CA IV is expressed in the endothelial cells of the brain capillaries (10). Mitochondrial CA V has been demonstrated in astrocytes and neurons throughout the central nervous systems of mice and rats (11).

To date, no CA has been identified in mammalian brain that can explain the effects of CA inhibitors in enhancing the extracellular alkaline shift seen in hippocampal slices after synaptic transmission (12–14). Such a CA has been postulated to have a modulating influence on excitatory synaptic transmission. The membrane CA XIV, the most recently discovered member of the α -CA gene family, is a membrane-spanning isozyme described independently by two groups (15, 16). Its deduced amino acid sequence showed an overall similarity of 29–46% to other active CA isozymes (15). Phylogenetic comparisons with amino acid sequences of other CAs place CA XIV in a cluster of extracellular CAs, clearly being most related to CA XII, followed by CA IX, VI, and IV. Northern blot analyses showed that CA XIV mRNA is expressed in human brain, heart, skeletal

muscle, and liver (14) as well as in mouse brain, kidney, heart, skeletal muscle, lung, and liver (16). Reasoning that CA XIV might be the long-suspected “extracellular” CA in mammalian brain, we developed antibody reagents for testing this hypothesis and used these reagents to define the immunolocalization of CA XIV in mouse and human brain.

Materials and Methods

Transfection of Chinese Hamster Ovary (CHO) Cells. A full-length cDNA expressing wild-type mouse CA XIV was isolated by using poly(A)-RNA from mouse kidney and PCR by using the primers described (16). To produce a secretory form of CA XIV, a stop codon was introduced at codon 279 (I279X). Full-length and truncated mouse CA XIV cDNAs were ligated into the mammalian expression vector pCXN as described recently for CA XII (17). These gene constructs were used to transfect CHO-K1 cells by electroporation. After selection in 400 μ g/ml G418 for 10 days, colonies were isolated and cultured. Clones secreting high levels of mouse CA XIV into the medium were identified by CA activity assay (18).

Antibodies. Mouse CA XIV secreted into the medium was affinity-purified by using a sulfonamide-agarose resin and used to prepare antibodies in two rabbits. Anti-human CA XIV antibody was raised against a polypeptide comprising the predicted 24 C-terminal amino acids of human CA XIV protein (KIRKKRLLENRKSVVFTSAQATTEA). The peptide was conjugated to keyhole limpet hemocyanin via a terminal cysteine and maleimide crosslinker. The polyclonal rabbit antibody was produced by Innovagen AB (Lund, Sweden).

SDS/PAGE and Western Blotting. All of the reagents for SDS/PAGE were from Bio-Rad. The electrophoreses were performed in a MiniProtean electrophoresis unit (Bio-Rad) under reducing conditions according to Laemmli (19), by using a 10% acrylamide separating gel and a 4% acrylamide stacking gel. Total cell protein (20 μ g) per lane from stably transfected CHO cells expressing wild-type mouse CA XIV was subjected to electrophoresis. The proteins were transferred electrophoretically from the gel to a poly(vinylidene difluoride) membrane (Millipore) in a NOVEX Blot Module (NOVEX, San Diego). After transblotting, the membrane was incubated first with TBST buffer (10 mM Tris·HCl, pH 7.5/150 mM NaCl/0.05%

Abbreviations: CA, carbonic anhydrase; CHO, Chinese hamster ovary.

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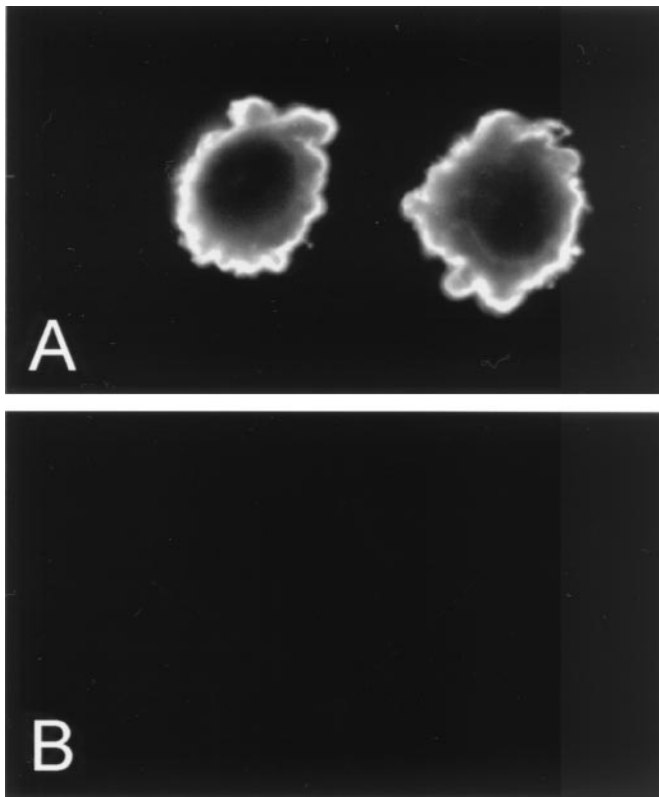


Fig. 1. A confocal laser scanning microscopy image of CA XIV in CHO cells transfected with mouse CA XIV full-length cDNA. (A) The anti-mouse CA XIV serum shows positive immunofluorescent reaction at the plasma membrane. (B) The cells labeled with normal rabbit serum. Original magnification: $\times 630$.

Tween 20] containing 10% (vol/vol) cow colostrum whey (BioTop, Oulu, Finland) overnight at 4°C and then with anti-mouse or anti-human CA XIV antiserum or preimmune serum diluted 1:2,000 in TBST buffer for 1 h at room temperature. The sheets were washed four times for 5 min with TBST buffer and incubated for 30 min at room temperature with alkaline phosphatase-conjugated goat anti-rabbit IgG (Bio-Rad) diluted 1:3,000 in TBST buffer. After washing three times for 5 min in TBST buffer, the polypeptides were visualized by using a chemiluminescence substrate (Bio-Rad).

Immunocytochemistry. The CHO cells transfected with mouse CA XIV cDNA were fixed with 4% (vol/vol) neutral-buffered formaldehyde for 15 min. Then they were rinsed with PBS and subjected to immunofluorescence staining that consisted of the following steps: (i) pretreatment of the cells with 0.1% BSA in PBS (BSA-PBS) for 40 min and rinsing in PBS; (ii) incubation for 1 h with the anti-mouse CA XIV antiserum or normal rabbit serum diluted 1:100 in 0.1% BSA-PBS; (iii) incubation with 1:30 diluted FITC-conjugated swine anti-rabbit IgG antibodies (Dakopatts, Glostrup, Denmark) in 0.1% BSA-PBS. The cells were washed three times for 2 min after the incubation steps. All incubations and washings were done in the presence of 0.05% saponin. The cells were viewed with a confocal laser-scanning microscope (Leitz). The specimens were excited with a laser beam at a wavelength of 488 nm, and the emission light was focused through a pinhole aperture. The full field was scanned in square image formats of 512×512 pixels, and built-in software was used to reconstruct the images obtained from the confocal sections.

Brain samples were obtained from adult mice that were killed under fentanyl fluanisone anesthesia. The brain halves were

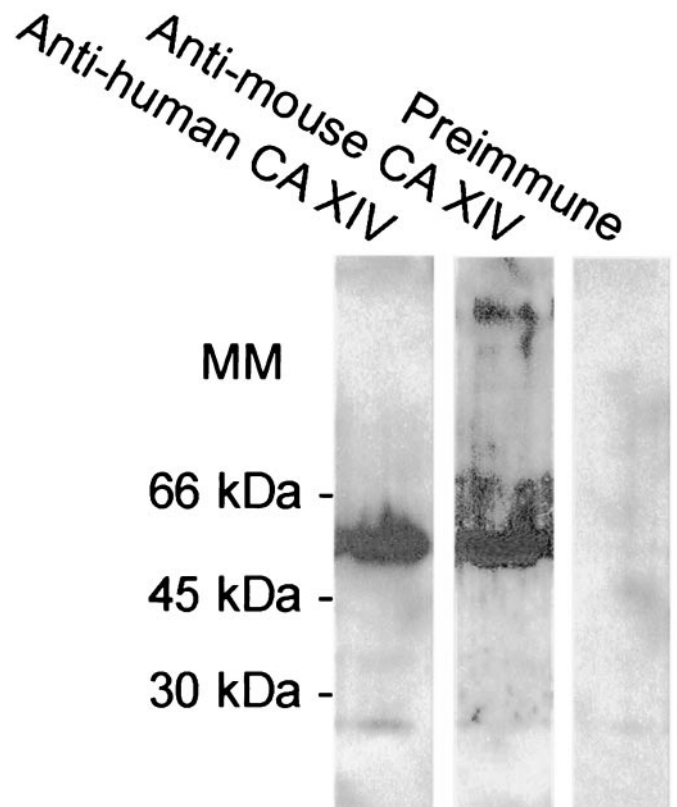


Fig. 2. Reactivity of the anti-human and anti-mouse CA XIV antisera with recombinant mouse CA XIV. An aliquot of 20 μ g of total cell homogenate from CHO cells transfected with mouse CA XIV full-length cDNA was applied to each lane. Both antisera identified a major polypeptide of 54 kDa on Western blots. No signal was seen when preimmune serum was used in place of the first antibodies. MM, molecular mass.

immersion-fixed in Carnoy's fluid (absolute ethanol/chloroform/glacial acetic acid, 6:3:1, vol/vol) for 6 h at 4°C. Then the samples were dehydrated and embedded in paraffin wax in a vacuum oven at 58°C. The tissues were sectioned into 11 coronal, 6 horizontal, and 8 sagittal layers. From each layer, we cut 10 sections (5 μ m each), and placed the sections on microscope slides. Pieces of human cerebral cortex, cerebellar cortex, hippocampus, choroid plexus, substantia nigra, and medulla oblongata were obtained from an autopsy of a 70-year-old donor who died because of a myocardial infarction. The brain shows no neuropathology. The human tissue samples were fixed in Carnoy's fluid as described above for the mouse tissues.

CA XIV was immunostained by the biotin-streptavidin complex method by employing the following steps: (i) pretreatment of the sections with undiluted cow colostrum whey for 40 min and rinsing in PBS; (ii) incubation for 1 h with the primary antiserum or preimmune serum diluted 1:100 in 1% BSA-PBS; (iii) incubation for 1 h with biotinylated swine anti-rabbit IgG (Dakopatts) diluted 1:300 in 1% BSA-PBS; (iv) incubation for 30 min with peroxidase-conjugated streptavidin (Dakopatts) diluted 1:500 in PBS; (v) incubation for 2 min in 3,3'-diaminobenzidine tetrahydrochloride (DAB) solution containing 9 mg of (Fluka) in 15 ml of PBS/5 μ l 30% (vol/vol) H_2O_2 . The sections were washed three times for 10 min in PBS after incubation steps ii, iii, and iv. All of the incubations and washings were carried out at room temperature, and the sections were finally mounted in Permount (Fisher). The sections were counterstained with hematoxylin. The stained sections were examined and photographed with a Nikon Eclipse E600 microscope.

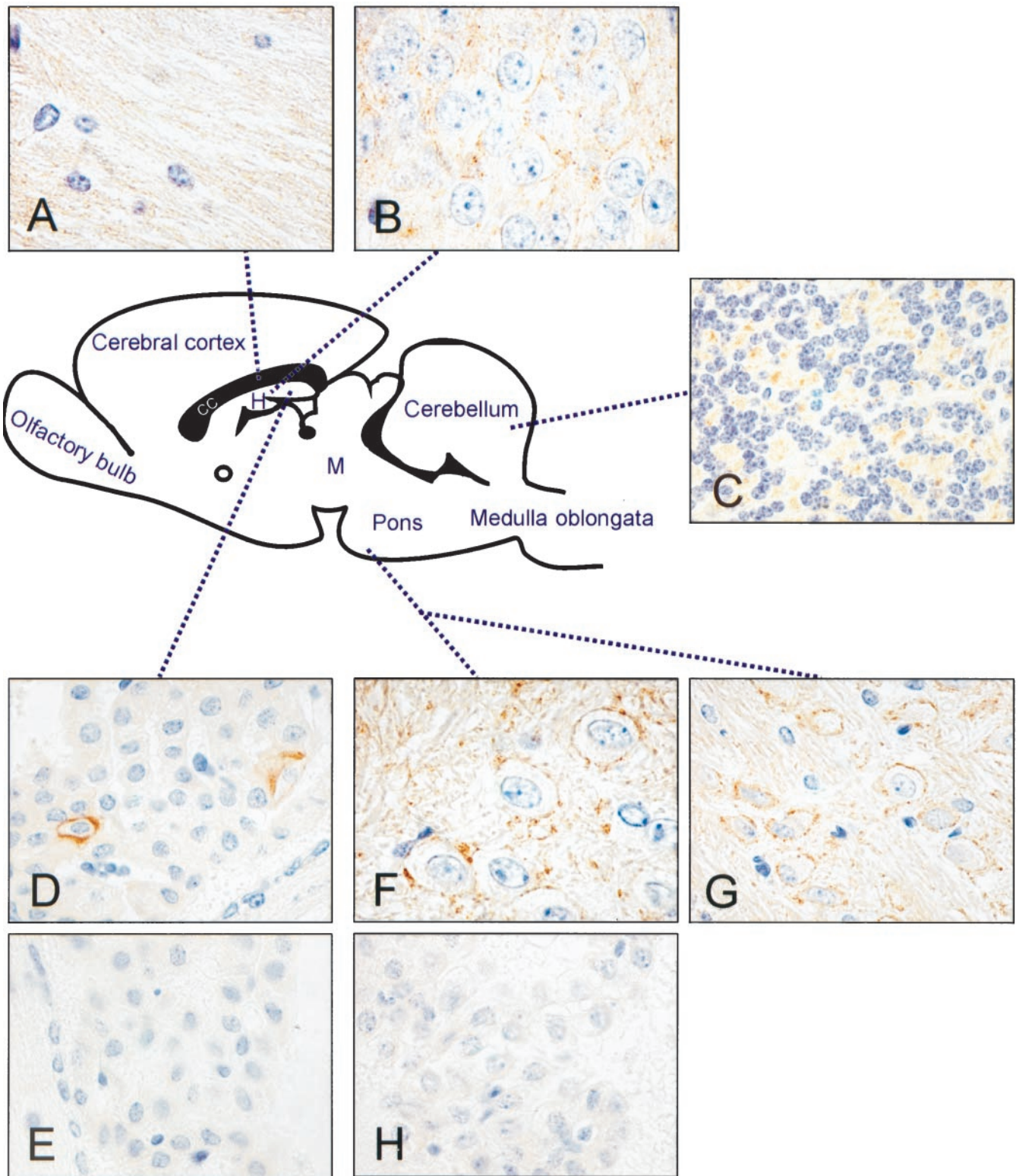


Fig. 3. Immunohistochemical staining of CA XIV in mouse brain. The schematic model indicates approximate anatomical sites which are shown in A–H. Strong positive staining by the biotin–streptavidin complex method, with anti-mouse CA XIV serum as first antibody, is seen in the large neuronal bodies and axons located in the basal part of pons (F and G). Other CA XIV-positive sites shown in this figure include corpus callosum (A), hippocampus (B), granular cell layer of cerebellum (C), and choroid plexus (D). The control sections of choroid plexus (E) and pons (H), with preimmune serum as first antibody, were negative. Original magnifications: A, G, and H, $\times 400$; B and F, $\times 500$; C, D, and E, $\times 250$. CC, corpus callosum; H, hippocampus; M, midbrain.

To confirm the immunostaining results by using another tissue processing technique, frozen sections of mouse brain were fixed in ice-cold acetone for 8 min. Then the sections were immuno-

stained by the immunofluorescence method employing the following steps: (i) pretreatment of the sections with undiluted cow colostrum whey for 40 min and rinsing in PBS; (ii) incubation

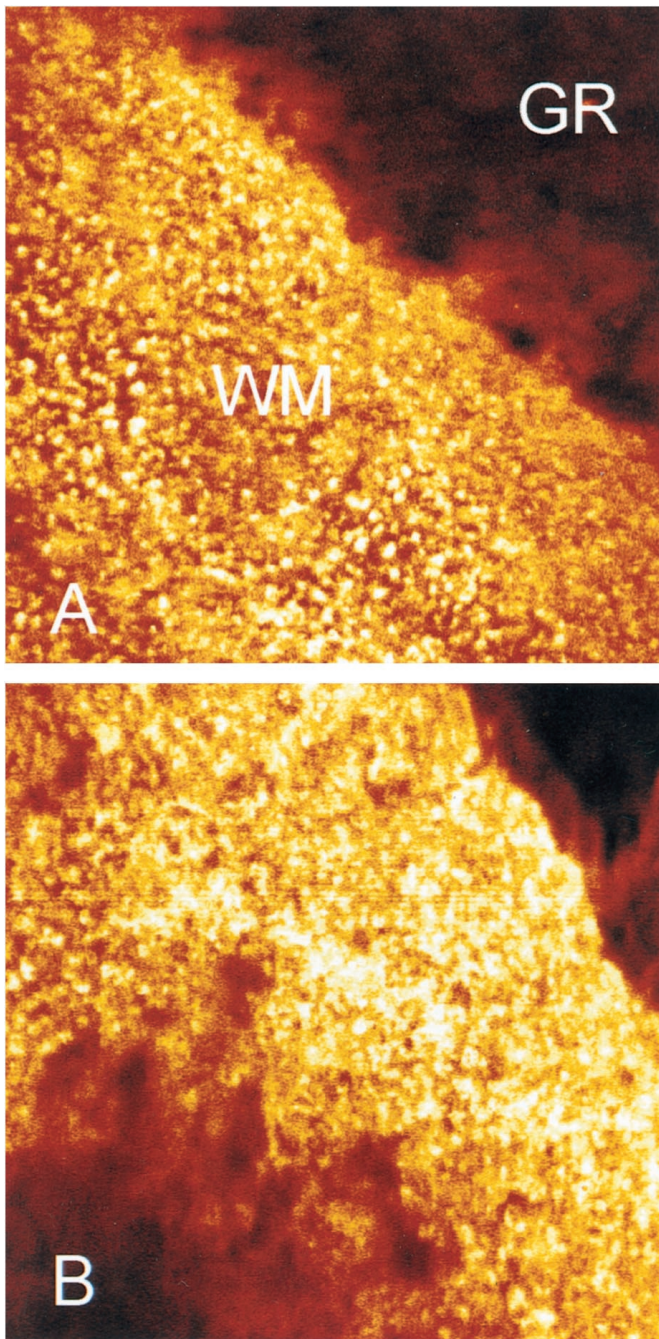


Fig. 4. Confocal laser scanning microscopy images of immunofluorescently labeled CA XIV in frozen sections of mouse brain. Punctate staining is located in the white matter (WM) of cerebellum (A) and anterolateral part of pons (B). The granular cell layer of cerebellum (GR) shows only a weak positive reaction. Original magnification: $\times 630$.

for 1 h with the anti-mouse CA XIV serum or preimmune serum diluted 1:100 in 1% BSA-PBS; (iii) incubation for 1 h with FITC-conjugated swine anti-rabbit IgG (Dakopatts) diluted 1:30 in 1% BSA-PBS. The sections were washed in PBS three times for 5 min after the incubation steps *ii* and *iii*. The sections were analyzed and photographed by using a confocal laser scanning microscope (Leitz CLSM). The antigenicity for CA XIV was preserved better in acetone-fixed samples. However, the paraffin-embedded samples fixed in Carnoy's fluid retained tissue morphology better for localization analyses.

Results

Characterization of Antibodies Raised Against Mouse and Human CA XIV. Two different rabbit antisera were used to study CA XIV in the mouse and human brain. The first was raised against recombinant mouse CA XIV produced in CHO cells. The second was raised against a synthetic peptide corresponding to the 24 C-terminal amino acids of the predicted human CA XIV protein, which differs in only 2 amino acids from the C-terminal 24 amino acids of mouse CA XIV. Fig. 1 shows a confocal laser scanning microscopy image of immunofluorescence staining for wild-type mouse CA XIV in transfected CHO cells. The anti-mouse CA XIV serum showed positive immunoreaction at the plasma membrane. The less intense intracellular signal probably represents the newly synthesized enzyme in the endoplasmic reticulum. The control immunostaining (with preimmune serum instead of a specific antibody) remained negative. Both antisera identified a strong 54-kDa polypeptide band of CA XIV on Western blots of CHO cells transfected with wild-type mouse CA XIV cDNA (Fig. 2).

Localization of CA XIV in Mouse Brain. For immunohistochemical analyses, mouse brain halves were dissected in 11 coronal, 6 horizontal, and 8 sagittal layers. From each layer, 10 5- μ m sections were cut and processed as described in *Materials and Methods*. The schematic model in Fig. 3 indicates approximate anatomical sites from which the sections were taken (see *A-H*). Immunostaining for CA XIV was found to be confined to distinct areas of the mouse brain. The most intense reactions were observed in the medulla oblongata and pons, in which the positive staining was located in the neuronal bodies and axons. Strong staining was also seen in the lateral and dorsal portions of the brainstem comprising cerebellar peduncles and in cerebellar white matter. In addition, the caudal area of mouse cerebellum showed staining in the neuropil of the granular cell layer, whereas the other layers of cerebellar cortex were negative. Fainter positive staining was observed in the large hippocampal neurons, in the nerve fibers of the corpus callosum and internal capsule, and in the molecular layer of the cerebral cortex. Occasional epithelial cells of the choroid plexus showed positive staining.

The positive staining was detected in both large neuronal bodies and axons. In neuronal bodies, it was most distinct at the plasma membrane. In cross sections of the nerve tracts, the staining was punctate, suggesting correspondence with axons. The axonal localization was also demonstrated in cerebellar white matter and medulla oblongata by using immunofluorescence staining (Fig. 4).

Localization of CA XIV in Human Brain. Human brain samples were also immunostained for CA XIV. Fig. 5 shows that CA XIV is expressed on the large neuronal bodies and on some axons in the medulla oblongata. This pattern of expression corresponded to that observed in the mouse. In the neuronal bodies, the positive signal was most prominent in the plasma membrane. The sample obtained from the cerebral cortex was negative, except for faint positive staining seen in some nerve fibers. No staining was detected in the sample of human cerebellar cortex.

Discussion

CA XIV is the most recently discovered member of the expanding α -CA gene family (15, 16). The human CA XIV gene was localized to chromosome 1q21. Prior Northern blotting results showed that CA XIV mRNA was expressed in mouse and human brain, in addition to multiple other organs. Hydrophathy plots with the deduced amino acid sequence and activity measurements suggested CA XIV was a transmembrane protein with an active extracellular CA domain like CA IX (20) and CA XII (17).

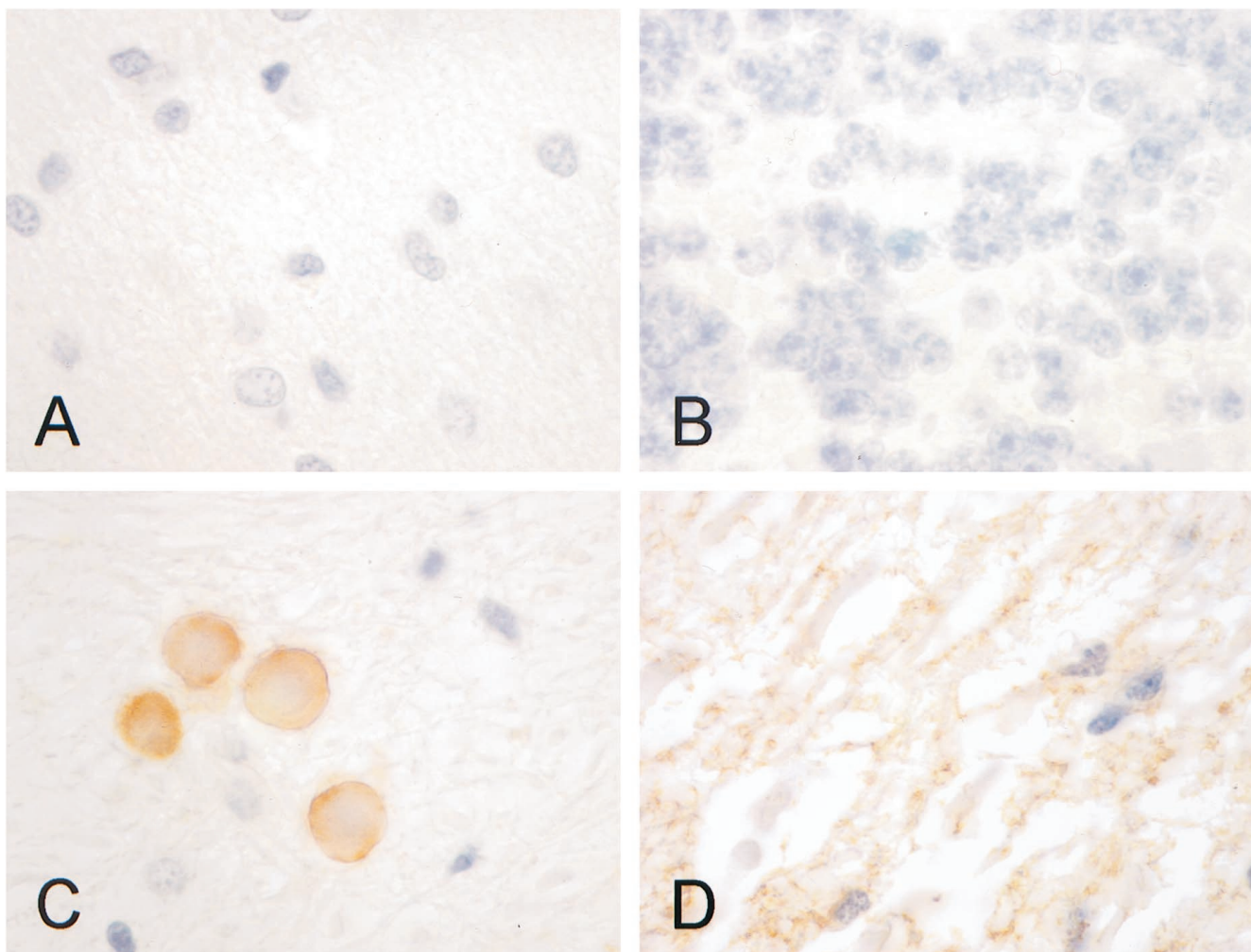


Fig. 5. Immunohistochemical staining of CA XIV in human cerebral cortex (A), granular cell layer of cerebellum (B), and medulla oblongata (C and D). Strong positive staining by the biotin–streptavidin complex method, by using anti-human CA XIV C-terminal peptide serum, is seen in the neuronal bodies and around nerve fibers in the medulla oblongata. Original magnification: $\times 500$.

These features suggested that CA XIV might be the extracellular CA that had been postulated to explain the effects of CA inhibitors in enhancing the extracellular alkaline shift after synaptic transmission (13, 14). Histochemical evidence had also suggested a CA activity on surfaces of neuronal cell bodies and axons (21), but no prior immunologic reagent had identified such an activity.

The present immunohistochemical data from CHO cells stably expressing recombinant CA XIV demonstrate that the enzyme is expressed on plasma membranes. Immunohistochemical staining of mouse and human brain sections showed that CA XIV has a distribution distinct from other CAs known to be expressed in the central nervous system (6–11). The strongest positive staining was observed in some nuclei and nerve tracts in the pons and medulla oblongata. In proximity to these strongly positive areas, the cerebellar peduncles and pyramidal tracts showed positive staining. From these studies, we conclude that CA XIV is expressed most highly in nuclei and nerve tracts that are associated with pontine, medullary, and hippocampal functions. One surprising finding was the immunohistochemical evidence that CA XIV was limited to certain areas of mouse and human brain, whereas the dot-blot analyses of mRNA expression in human brain showed more widespread expression. This discrepancy may reflect differences in sensitivity of the two methods of detection. It may also reflect real differences in localization of

CA XIV mRNA and protein. Translational regulation of CA XIV may lead to greater amounts of expressed protein in some areas of brain. Ribosomal protein L4 levels were shown to be regulated by translational control (22). Transcription-independent, translational-dependent neurite extension occurs in adult rat sensory neurons after sciatic nerve crush and in nerve growth factor-differentiated PC12 cells whose neurites have been sheared mechanically.

What is clear is that CA XIV is located on the plasma membrane of some neurons and on axons of both mouse and human brain. In this location, it could explain the prior histochemical stains interpreted to reflect a CA activity on the membranes of neurons and axons as well as the observations that CA inhibitors enhance the extracellular alkaline shift after excitatory synaptic transmission (12–14). It was the latter observations that suggested an important role for an extracellular CA in modulating the response to excitatory stimuli at synaptic termini. Of the many CAs characterized thus far, CA XIV seems the best candidate for the extracellular CA that mediates this modulation.

Before brain CAs were characterized fully, neurological effects of CA inhibitors were attributed to secondary effects of inhibition of erythrocyte CAs (23). Later, it was suggested that inhibition of CA II in brain might account for some of the neurological side effects of CA inhibitors (24). The fact that CA

II is limited to oligodendrocytes and a few astrocytes and to the choroid plexus makes it less likely that inhibition of CA II is the explanation for some central effects of these inhibitors, such as their effects on neuroexcitation and seizure activity. In fact, Tong *et al.* (14) recently demonstrated that absence of CA II in the hippocampal slices from CA II-deficient mice has no effect on the alkaline shift after excitatory stimulation. The finding that CA IV is located on the luminal surface of cerebral capillaries, and associated with the blood–brain barrier, made it another potential candidate to explain some of the effects of CA inhibitors that influence brain function (10). More recently, CA V (particularly CA Vb) was identified in rat and mouse neurons and glial cells and became still another candidate to explain some neurological side effects of CA inhibitors (11). The findings

reported here showing CA XIV expression on neuronal membranes and axons in specific areas of brain including hippocampus make it an especially strong candidate for the extracellular CA activity, which accounts for the observations that CA inhibitors amplify the extracellular alkaline shift after excitatory stimulation in brain. We propose that CA XIV is the major target of these inhibitors and thus an important isozyme in modulating these changes after excitatory synaptic transmission. Either an isozyme-specific inhibitor or an inhibitory antibody that selectively inhibits CA XIV would allow a direct test of this hypothesis.

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