Expression of membrane-bound carbonic anhydrase isozyme XII in mouse and rat tissues

Master's thesis
Institute of Medical Technology
University of Tampere
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Piia Halmi

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Tiivistelmä

Tutkimuksen tausta ja tavoitteet: Hiilihappoanhydraasi XII (CA XII) on solukalvoon kiinnittyvä isoentsyymi, joka ilmentyy joissakin ihmisen normaaleissa soluissa sekä yliekspressoituu joissakin syöpäsoluissa. CA12 geenin on todettu yliekspressoituvan joissakin syöpäkudoksissa hypoksian johdosta. Tässä tutkimuksessa selvitettiin CA XII:n ilmetymistä hiiren ja rotan elimistössä. Tutkimuksen tavoitteena oli tutkia ensin CA XII esiintymistä hiiren kudoksissa, ja myöhemmin keskittyä ilmentymisen tarkempaan tutkimiseen aivokudoksissa tunnetulla rottamallilla.

Tutkimusmenetelmät: Lähetti-RNA:n transkriptiota tutkittiin hiiren kudoksissa PCR:n avulla, ja rotan kudoksissa käänteiskopiointi-PCR:n (RT-PCR) avulla. Hiiren CA XII –proteiinia tutkittiin immunohistokemialla (biotiini-strepavidiini-menetelmä). Rotan aivokudoksia tutkittiin kainaattihapon aiheuttaman epilepsikohtauksen jälkeen sekä in situ -hybridisaation että northern blot -menetelmän avulla. In situ -hybridisaatiolla paikannettiin ilmentyminen, ja northern blot -menetelmällä kvantitoitiin ilmentymistä.

Tutkimustulokset: CA XII mRNA ilmentyy mm. hiiren munuaisissa, aivoissa, keuhkoissa, kivespusseissa ja sikiöissä. CA XII -proteiinilla on erittäin rajoittunut ilmentymiskenttä (munuainen, paksusuoli). Rotan aivoissa CA XII mRNA ilmentyy lähinnä choroid plexus:ssa ja aivokuoressa. Kainaattihappo stimuloi CA XII ilmentymistä kortikaalipinnalla.

Johtopäätökset: Runsas CA XII ekspressio hiiren munuaisissa ja paksusuolessa luultavasti selittää proteiinin merkitystä sekä kehon ioni- että pH-tasapainossa. Kainaattihapon aiheuttaman stimuloinnin fysiologinen merkitys jäi epäselväksi, mutta CA XII:n runsas ilmentyminen choroid plexus:en alueella kertoo luultavasti nalogisesta toiminnasta CA II:n kanssa. CA II osallistuu aivoselkäydinnesteen erityksen.

MASTER´S THESIS

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Abstract

Background and aims: Carbonic anhydrase XII (CA XII) is a membrane-bound isozyme expressed in some normal human tissues, upregulated in some cancers, and is showed to be a hypoxia-inducible gene product. The expression of CA XII mRNA has been demonstrated in mouse kidney. This study concentrated on the research of the expression of CA XII in all mouse tissues, and later on in rat brain tissues.

Methods: mRNA transcription was studied by PCR in mouse tissues and by reverse transcriptase-PCR (RT-PCR) in rat tissues. CA XII protein in mouse tissues was studied by Immunohistochemistry (biotin streptavidin complex method). The studies on rat brain tissues were made by in situ hybridization and northern blot methods after kainic acid-induced status epilepticus. In situ hybridization showed the location of the expression where as northern blot served as a measure of quantity of the expression.

Results: CA XII mRNA is expressed in mouse kidney, brain, lung, testis and embryos. In embryos the expression became stronger with increasing age indicating developmental regulation. CA XII protein has a very limited expression distribution (mouse kidney and colon). In rat brain tissues CA XII mRNA is expressed mainly in dentate ganule cells, cortex and choroid plexus. Kainic acid stimulated the expression throughout the cortical layer.

Conclusions: The high expression of CA XII in mouse kidney and colon suggests a role for CA XII in the maintenance of body ion and pH homeostasis in the mouse. Kainic acid stimulates CA XII expression throughout the cortical layer. The physiological significance of the observed cortical induction of CA XII remains obscure, but the high expression of CA XII in the choroid plexus suggests an analogous role for this membrane-bound isozyme. CA II is known to participate in CSF secretion.

CONTENTS

ABBREVIATIONS

AE	anionic exchanger
AQP	aquaporin
ARNT	aryl hydrocarbon receptor nuclear translocator
BBB	bloob-brain barrier
BSA	bovine serum albumin
CA	carbonic anhydrase
CA12	*carbonic anhydrase 12* (refers particularly to the human gene)
Car7	*carbonic anhydrase 7* (refers particularly to the mouse gene)
CA-RP	carbonic anhydrase related protein
CD	collecting duct
cDNA	complementary deoxyribonucleic acid
CNS	central nervous system
CSF	cerebrospinal fluid
Da	Dalton
DAB	3,3'diaminobenzidine tetrahydrochloride
DEPC	diethylpyrocarbonate
DNase	deoxyribonuclease
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EEG	electroencephalogram
G3PDH	glyceraldehyde 3-phosphate dehydrogenase
GABA	γ-aminobutyric acid
GL	glomerulus
Gly	glysine
GPI	glycosyl phosphatidylinositol
HIF	hypoxia inducible factor
His	histidine residue
IEG	immediate-early gene
mRNA	messenger ribonucleic acid

NMDA	N-methyl-D-aspartate
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PCT	proximal convoluted tubule
pVHL	von Hippel-Lindau protein
RNA	ribonucleic acid
RNAse	ribonuclease
RPTP	receptor protein tyrosine phosphatase
RT-PCR	reverse transcriptase polymerase chain reaction
SDS	sodium dodecyl sulphate
Ser	serine
Ub	ubiquitin
VBC	VHL/Elongin B/Elongin C
VEGF	vascular endothelial cell growth factor
VHL	von Hippel-Lindau

1. INTRODUCTION

Carbonic anhydrases (CAs) are a family of zinc metal enzymes which catalyze the reversible spontaneous hydration of carbon dioxide (CO_2) (Sly & Hu, 1995). There are at least 13 known α-CAs which are characterized in animal kingdom and three CA-related proteins which lack enzymatic activity. A recent study shows a novel member of this gene family, carbonic anhydrase XV, which seems to exist as a non-processed pseudogene in humans and chimpanzees (Hilvo et al., 2005).

Carbonic anhydrase XII, CA XII, is a 354-amino acid polypeptide type I transmembrane protein with its active extracellular domain containing three zinc-binding histidine residues which can be found in active CAs and two potential sites for asparagine glycosylation. CA XII protein sequence has a sequence identity of 30-42 % to other CAs (Türeci et al., 1998) and it exists as a dimer in both solution and the crystal. The *CA12* gene has been identified as a von Hippel-Lindau target gene, suggesting a potential role for CA XII in von Hippel-Lindau carcinogenesis (Ivanov et al., 1998). Recently, CA XII has been found to be a hypoxia-inducible protein (Ivanov et al., 2001; Wykoff et al., 2001; Watson et al., 2003), possibly explaining its upregulation in certain tumors. These recent reports suggest that CA XII may be an excellent marker for hypoxia in tumors.

CA XII has a wide distribution spectrum in normal tissues (Ivanov et al., 1998; Karhumaa et al., 2000; Kivelä et al., 2000; Parkkila et al., 2000; Türeci et al., 1998). CA XII mRNA is expressed in numerous human tissues such as aorta, bladder, brain, colon, esophagus, kidney, lung, mammary gland, ovary, prostate, pancreas, rectum, testis, trachea and uterus (Türeci et al., 1998; Ivanov et al., 1998; Kivelä et al., 2000). In the brain *CA12* gene is expressed only in the corpus striatum (caudate nucleus and putamen) (Ivanov et al., 1998; 2001). Immunohistochemical studies show CA XII expression in the human reproductive tissues, colon, mesothelial cells and the coelomic epithelium of the body cavity, and kidney, sweat glands of the skin, epithelium of the breast, salivary glands, upper respiratory system, nose, and pancreas. Limited expression can be found also in the prostate, vas deferens, and transitional mucosa of the renal pelvis. In the

gastrointestinal tract CA XII expression is limited to the surface epithelium of the large intestine. In the nervous system CA XII immunoreactivity is found in the choroid plexus, in limited numbers of ganglion cells of the cortex, in the posterior lobe of the pituitary glands, and in the remnant of Rathke's pouch (Ivanov et al., 2001; Karhumaa et al., 2000; 2001; Kivelä et al., 2000; Parkkila et al., 2000).

The expression of *CA12* in multiple cancers has been shown to be in high level (Ivanov et al., 2001) but its function in normal and malignant tissues is not yet exactly known. The CA XII overexpression has been shown in some human renal cancer cells (Türeci et al., 1998). Based on its expression pattern CA XII could serve as a biomarker for some malignant tumors (non-small cell lung carcinoma) and could be considered a potential target for novel therapeutic applications (Ivanov et al., 1998).

This study is focused on the expression and distribution of CA XII in mouse tissues and rat brain tissues. We also evaluate CA XII expression in the rat brain after kainic acid induced epileptic seizures.

2. REVIEW OF THE LITERATURE

2.1. ENZYMES

Enzymes are special cases among other proteins in that they bind and chemically transform (catalyze reactions) other molecules. Their catalytic power is often far greater than that of synthetic or inorganic catalysts. The molecules acted upon by enzymes are called reaction substrates, and the ligand-binding site is called the active site or catalytic site. Enzymes have a high degree of specificity for their substrates. They accelerate chemical reactions, and function in aqueous solutions under very mild conditions of temperature and pH. The study of enzymes is of great importance because a deficiency or total absence of one or more enzymes or their excessive activity may cause various diseases (Nelson & Cox, 2000).

Some enzymes do not require any chemical groups for activity other than their amino acid residues, while others require an additional chemical component called a cofactor (one or more inorganic ions, e.g. Fe^{2+}, Mn^{2+}, or Zn^{2+}) or a complex organic or metalloorganic molecule called a coenzyme. There are even some enzymes which require both a coenzyme and one or more metal ions for activity. In the case of carbonic anhydrases, the enzymes require Zn^{2+} ion in their catalytic site. Catalytically active enzyme is called a holoenzyme, and its protein part apoenzyme or apoprotein. A coenzyme or a cofactor bound tightly or covalently to the enzyme protein is called a prosthetic group. Enzyme activity can be altered also by modifying the enzyme covalently by phosphorylation or glycosylation (Nelson & Cox, 2000).

2.2. CARBONIC ANHYDRASES

The carbonic anhydrases catalyze the reversible spontaneous hydration of carbon dioxide (CO_2) with high efficiency, a reaction which underlies many diverse physiological processes in animals, plants, archaebacteria, and eubacteria such as photosynthesis, respiration, renal tubular acidification, and bone resorption. The carbon dioxide produced

by oxidation of organic fuels in mitochondria is hydrated to form bicarbonate (HCO_3^-) by carbonic anhydrase (figure 2.1.).

$$CO_2 + H_2O \Leftrightarrow H_2CO_3 \Leftrightarrow H^+ + HCO_3^-$$
$$HCO_3^- \Leftrightarrow H^+ + CO_3^{2-}$$

Figure 2.1. Hydration of carbon dioxide (CO_2) to bicarbonate (HCO_3^-).

Carbon dioxide is not very soluble in aqueous solution, and it would form bubbles of CO_2 in the tissues and blood if it were not converted to bicarbonate. The hydration of CO_2 to bicarbonate results in an increase in the H^+ concentration in the tissues and therefore a decrease in pH. Bicarbonate in the erythrocytes reenters the blood plasma for transport to the lungs (figure 2.2.).

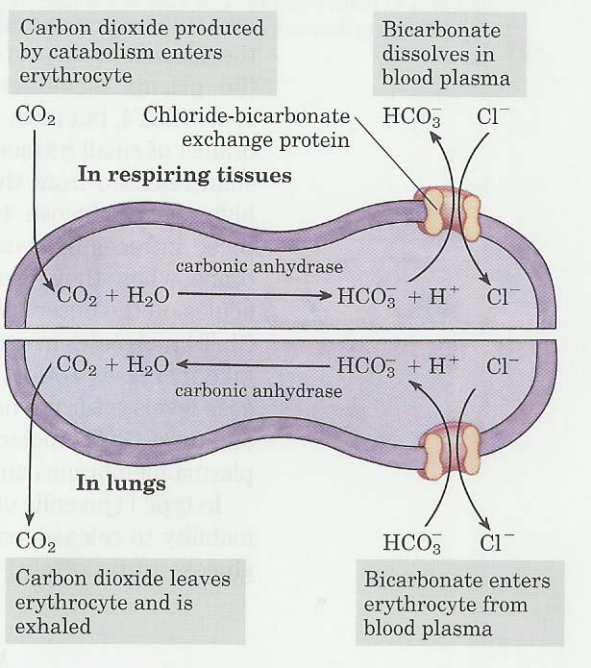

Figure 2.2. The function of chloride-bicarbonate exchanger and carbonic anhydrase in the erythrocyte membrane (Modified from Nelson & Cox, 2000).

Compared to CO_2, HCO_3^- is much more soluble in blood plasma. Therefore, the generation of HCO^-_3 ions increases the capacity of the blood to carry CO_2 from the tissues to the lungs. In the lungs HCO_3^- reenters the erythrocyte and is converted to CO_2, which is released into the lung space and exhaled. The binding of oxygen by hemoglobin

is influenced by pH and CO_2 concentration, so the interconversion of CO_2 and bicarbonate is of great importance to the regulation of oxygen binding and release in the blood. About 20% of the total CO_2 and H^+ formed in the tissues is transported by haemoglobin to the lungs and kidneys. The binding of these ions is inversely related to oxygen binding. At the low pH and high CO_2 concentration (peripheral tissues) the affinity of haemoglobin for oxygen decreases and oxygen is released. In the capillaries (lung tissues) CO_2 excretion causes the raise of pH, and the affinity of haemoglobin for oxygen increases. More oxygen is transported to the peripheral tissues (Nelson & Cox, 2000).

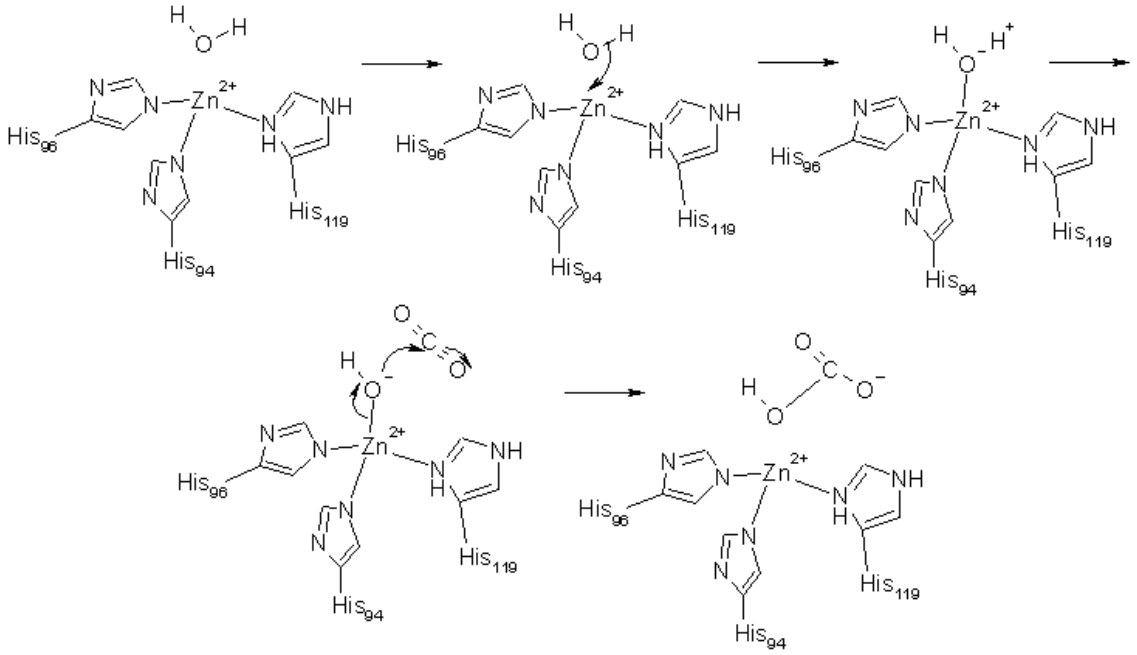

Figure 2.3. Oxidation of carbon dioxide (CO_2) to form bicarbonate (HCO_3^-) with the help of a zinc ion. Zinc is bound to three histidines in carbonic anhydrase. Water is ionised to a hydroxide ion and this is stabilised by the Zn^{2+} ion. CO_2 then enters the active site and is attacked by OH^-, forming a carbonate ion, which is then released, regenerating the enzyme.

All enzymatically active carbonic anhydrases contain a zinc ion (Zn^{2+}), which is critical for the catalytic activity (figure 2.3.). The zinc ion is coordinated by three histidine residues (Stams & Christianson, 2000). The transfer of H^+ from the the zinc-bound water molecule to the solution generates OH^-. The central catalytic step is a reaction between

this zinc-bound OH^- ion and CO_2 leading to a HCO_3^- ion which the water molecule displaces from the metal ion (Lindskog & Silverman, 2000; Supuran, 2004).

Carbonic anhydrases are clustered into three distinct gene classes (α, β, and γ) which have evolved independently and have no sequence homology (Hewett-Emmet & Tashian, 1996). All the 13 characterized CAs of the animal kingdom belong to a single gene family of the α-carbonic anhydrases (α-CAs). These isozymes have been found in all mammalian tissues and cell types and they show characteristic cellular localization; they are found in cytosol (CA I, CA II, CA III, CA VII and CA XIII) (Sly and Hu, 1995; Earnhardt et al., 1998; Lehtonen et al., 2004), mitochondria (CA VA and CA VB) (Fujikawa-Adachi et al., 1999), secretory granules (CA VI) (Murakami & Sly, 1987), and plasma membrane (CA IV, CA IX, CA XII, CA XIV and CA XV) (Zhu and Sly, 1990; Pastorek et al., 1994; Türeci et al., 1998; Ivanov et al., 1998; Mori et al., 1999; Hilvo et al., 2005) (table 2.1.).

Table 2.1. Carbonic anhydrases and their subcellular localization.

Carbonic anhydrase	Subcellular localization
I	cytoplasmic
II	cytoplasmic
III	cytoplasmic
IV	membrane-bound
VA	mitochondrial
VB	mitochondrial
VI	secreted
VII	cytoplasmic
IX	transmembrane
XII	transmembrane
XIII	cytoplasmic
XIV	transmembrane
XV	membrane-bound

There are also other known inactive isoforms which have homologous domains to active carbonic anhydrases (CA-RP VIII, CA-RP X, CA-RP XI, RPTP- β and RPTP- γ) which do not appear to have any activity similar to earlier ones. This is due to changes in the active site histidine (His) residues which are critical for carbon dioxide hydration catalysis (zinc ion is not bound when His residues are missing). These isoforms are widespread in mammalian body tissues (Nishimori, 2004; Taniuchi et al., 2002a; 2002b).

The α-CA isozymes differ in their tissue distribution, subcellular localization, and kinetic properties. Some isozymes such as CA II are expressed in a number of different tissues, whereas others (e.g. CA VI, IX and XIV) show a more limited distribution. All active isozymes are expressed in the alimentary tract, although the cellular localization is unique for each isozyme (Parkkila et al., 1994b; Fleming et al., 1995; Sly and Hu, 1995; Parkkila & Parkkila, 1996; Pastoreková et al., 1997; Kivelä et al., 2000).

Distinct α-CAs can be found in protostomes (*Drosophila melanogaster, Caenorhabditis elegans, Caenorhabditis briggsae, Galeocerdo cuvieri*). Several plant chloroplast CAs and certain eubacteria (*Escherichia coli* and *Synechococcus*) CAs have been shown to belong to a distinct gene family, β-carbonic anhydrases (β-CAs) (Fukuzawa et al., 1992; Guilloton et al., 1992; 1993). The third distinct gene family is represented by γ-CAs which includes at least the isoforms of archaebacterium *Methanosarcina thermophila* (Alber & Ferry, 1994). This division of CA isoforms into three separate classes is only a simple assumption because the plant *Arabidopsis* for example has homologues of all of these three enzyme families (Hewett-Emmett & Tashian, 1996).

2.2.1. α-CARBONIC ANHYDRASES

2.2.1.1. Cytoplasmic carbonic anhydrases

The **carbonic anhydrases I and II** are both quite widely expressed cytoplasmic isozymes. The *CA1*, *CA2* and *CA3* genes exist in a cluster on the chromosome 8q22. CA I is expressed in the α-cells of the endocrine Langerhans' islets (Parkkila et al., 1994b). It is a low activity isozyme (15 % of that of CA II) which has a molecular weight of 30,000

Da. CA I is expressed at low levels in the A cells of Langerhans islets, the epithelium of the large intestine, corneal epithelium, the lens of the eye, the placenta and foetal membranes (Muhlhauser et al., 1994; Parkkila et al., 1994; Sly & Hu, 1995), and overexpressed in chronic myeloproliferative disorders (Bonapace et al., 2004a). The CA I and II are expressed in human colonic mucosa in the non-goblet columnar cells lining the main lumen in the upper half of the crypts (Bekku et al., 1998; Davenport & Fisher 1938; Davenport, 1939; Lönnerholm et al., 1985; O'Brien et al., 1977; Sato et al., 1980; Parkkila et al., 1994b). The expression has been demonstrated to increase gradually over time during differentiation of the mucosal cells, and therefore they could function as useful markers for the differentiation of enterocytes in the colonic mucosa. CA I is five times as abundant as CA II in human erythrocytes. Because CA I is a low activity isozyme, it contributes only about 50% of the total activity in these cells, which could explain why CA II deficient people do not have defects in erythrocytes (Dodgson et al., 1988).

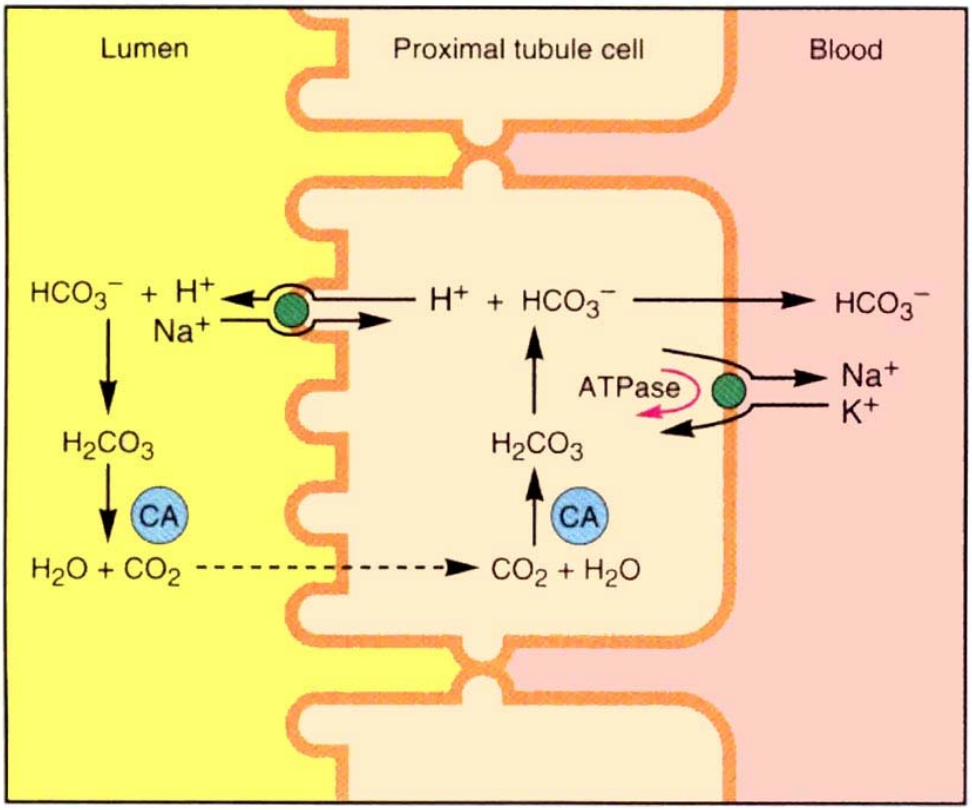

Figure 2.4. Function of carbonic anhydrase II in the proximal tubule cell in the kidney (Modified from Fox, 1996).

Carbonic anhydrase II is a best known isoenzyme and is located almost in all organs. It has a molecular weight of 30,000 Da and is highly expressed in the intercalated cells of the late distal tubule, the collecting tubule, and the collecting duct of the kidney and some expression has been reported in the loop of Henle, the proximal tubules (figure 2.4.), and the principal cells of the collecting ducts (Brown et al., 1983; Brown & Kumpulainen, 1985; Holthöfer et al., 1987; Lönnerholm et al., 1986; Lönnerholm & Wistrand, 1984; Parkkila et al., 1994b; Sato & Spicer, 1982; Spicer et al., 1982; Spicer et al., 1990). Sly et al. (1983) reported renal tubular acidosis in patients with CA II-deficiency syndrome. CA II-deficient mice produced by chemical mutagenesis also had impaired renal acidification (Lewis et al., 1988). More than 95 per cent of renal CA activity is cytosolic and corresponds to CA II, while 3-5 per cent is membrane associated (McKinley & Whitney, 1976; Wistrand & Kinne, 1977). In the hepatic bile ducts, CA II facilitates the alkalization of the bile (Parkkila & Parkkila, 1996) and is involved in bile acidification occurring in the gallbladder (Juvonen et al., 1994). CA II is present in the human epididymis and ductus deferens where it could be involved in the acidification of the epididymal fluid. It seems to be abundant in the brush border regions of the surface epithelium. The bicarbonate of the ejaculate is produced by CA II in the epithelium of ductus deferens and seminal vesicle. The bicarbonate ions present in the seminal fluid may induce sperm motility in the vagina and cervix and also buffer the low pH of the vaginal milieu. The pH of the CSF produced in the choroid plexus is regulated by CA II. CA II catalyzes also the hydration of CO_2 to HCO_3^- in erythrocytes and the HCO_3^- production to saliva (Chedwiggen & Carter, 2000).

In 1974 Garg detected for the first time CA III in rat liver. This very low activity isozyme, **CA III**, is expressed also in adipocytes and skeletal muscle (Jeffery et al., 1980; Kim et al., 2004a). It is one of the first isozymes detected by specific antibodies (Spicer et al., 1979; 1982; 1990). It functions in an oxidizing environment and it is the most oxidatively modified protein in the liver known so far (Cabiscol & Levine, 1995). CA III may provide protection from oxidative damage and may serve as a useful marker protein to investigate *in vivo* the mechanisms contributing to oxidative damage in the liver (Parkkila et al., 1999). Low levels of free radicals in cells over-expressing CA III may

affect growth-signalling pathways (Räisänen et al., 1999). The knock-out mouse model generated by Kim et al. (2004a) showed no morphological neither physiological abnormalities, and CA III deficient mice studied by Zimmerman et al. (2004) suggested a anti-oxidative role for this protein in the skeletal muscle.

The carbonic anhydrase VII is the most highly conserved α-CA. The sequence identity is ~95% between human and mouse homologues which suggests an important biological role for this enzyme (Sly & Hu, 1995). The *CA7* gene has been localized to the chromosome 16q22-23 (Montgomery et al., 1991). The CA VII enzyme has been expressed in *E. coli* and shown to possess carbon dioxide hydrase activity (Lakkis et al., 1996). *In situ* hybridization has demonstrated CA VII expression in mouse brain at high level in the Purkinje cells, in the granular and molecular layers, at the pial surface and in the large neurons throughout the cortical layer, in the medial habenulae, in neurons of the thalamus, strongly expressed in the hippocampal formation, specifically in the pyramidal cells of Ammon's horn and in the granular cells of the dentate gyrus, and in the choroid plexus and cerebrospinal fluid-containing channels (Lakkis et al., 1997). This distribution of mRNA in the mouse brain could suggest a non-specific but generalized function for CA VII in the mouse brain. CA activity plays an important role in cerebrospinal fluid production and in regulation of its ionic constituents and pH. The studies showed a very strong expression in neurons, hence the CA VII could be important in maintaining the metabolic activity of the neurons by the elimination and transport of CO_2 produced by glycolysis and it could effect on neuroexcitation and susceptibility to seizures in many distinct ways. CA VII can be important in maintaining different membrane transport processes by facilitation of CO_2 transport and regulation of transmembrane fluxes (correct distribution of chloride ions). Some connection between CA activity in the dorsal root ganglia with the cytochrome oxidase activity has been commented. This could effect on the electrical activity of the neurons and their energy requirements. High concentrations of CA are probably necessary in the growth and maturation of neurons. A peripheral nerve injury reduces CA enzyme content or its activity in dorsal root ganglion cells (Lakkis et al., 1997). Ruusuvuori et al. (2004) suggest a function for CA VII in a developmental process enabling synchronous firing of CA1 pyramidal neurons.

A novel carbonic anhydrase XIII, **CA XIII**, has been recently characterized (Lehtonen et al., 2004). CA XII mRNA is expressed in the human thymus, small intestine, spleen, prostate, ovary, colon, and testis and in the mouse spleen, lung, kidney, heart, brain, skeletal muscle, and testis. Distribution of CA XIII shows similarities with that of other cytosolic isozymes. Computer modelling of CA XIII structure revealed that it is a globular molecule with high structural similarity to cytosolic isozymes, CA I, II, and III. Furthermore, kinetic studies have demonstrated catalytic activity similar to mitochondrial CA V and cytosolic CA I.

2.2.1.2. Mitochondrial and secretory carbonic anhydrases

Dodgson (1991) described the expression of CA V for the first time in the rat liver and kidney, and in the mitochondria of the liver and skeletal muscle of guinea pigs. Later studies have revealed that mammalian tissues contain two different mitochondrial CA isoforms, **CA VA and CAVB** (Fujikawa-Adachi et al., 1999). CA VA is expressed mainly in the liver, whereas CA VB has a wide expression distribution in many tissues except in the liver (Shah et al., 2000). The expression of human CA V cDNA in COS-7 cells produced a 34,000 DA precursor and 30,000 Da mature enzymes (Nagao et al., 1993). CA V is the second isozyme described in the endocrine pancreas, where its expression is solely confined to the β-cells (Parkkila et al., 1998). It has been proposed that mitochondrial CA regulates insulin secretion by providing bicarbonate ions for the pyruvate-malate shuttle operating in these cells. CA V is expressed also in brain tissue and in the gastrointestinal tract (Saarnio et al., 1999; Sato et al., 2002).

The mammalian liver expresses high levels of mitochondrial CA V and it has been implicated in two metabolic processes in the mitochondria of hepatocytes: ureagenesis and gluconeogenesis. CA V would supply bicarbonate for the first urea cycle enzyme, carbamyl phosphate synthetase I in ureagenesis and for pyruvate carboxylase in gluconeogenesis (Dodgson, 1991). CA V could have an important role also in lipogenesis ()pyruvate carboxylation) (Lynch et al., 1995; Hazen et al., 1996). CA inhibitors have

been observed to retard both of these processes in the livers of guinea pigs and rats (Dodgson et al., 1983; Metcalfe et al., 1985; Dodgson, 1991).

The only known secretory carbonic anhydrase of the CA gene family is **CA VI**. It is secreted from the salivary glands (Fernley et al., 1979; Feldstein & Silverman 1984; Murakami & Sly 1987; Kadoya et al., 1987). The amino acid sequence of sheep CA VI has been reported (Fernley et al., 1988), and Aldred et al. (1991) have determined the cDNA sequence of human CA VI. A probable stabilization of CA VI in the environment of the alimentary tract is caused by an intramolecular disulfide bond formed by two cysteine residues (Fernley et al. 1988; Aldred et al. 1991; Parkkila et al. 1997). CA domain of CA VI is highly homologous to four other CAs (CA IV, CA IX, CA XII and CA XIV) and they form together a group of extracellular CAs (Fujikawa-Adachi et al., 1999b; Mori et al., 1999).

The molecular weight of CA VI varies depending on a species between 39 and 46 kDa (Parkkila & Parkkila, 1996). CA VI is produced in the serous acinar cells of the parotid and submandibular glands (Kadoya et al., 1987; Parkkila et al., 1990). The secretion of CA VI is controlled by autonomic nervous system (Fernley, 1991), and it follows circadian period (Kivelä et al., 1997; Parkkila et al., 1993; Parkkila et al., 1995). CA VI protects teeth from caries by accelerating the neutralization of the protons produced by cariogenic bacteria (Kivelä et al., 1999) and it is also involved in the neutralization processes in the upper gastrointestinal tract (Parkkila et al., 1997) and pancreas (Fujikawa-Adachi et al., 1999a). CA VI has been suggested to be linked with taste function (Thatcher et al., 1998) and furthermore as a trophic factor for the taste bud stem cells (Henkin et al., 1999b). Karhumaa et al. (2001) has reported the presence of CA VI in milk, and Kimoto et al. (2004) in the mouse nasal gland. CA VI has been suggested to have a mucosa-protective role also in the respiratory tract (Leinonen et al., 2004).

2.2.1.3. Membrane-associated carbonic anhydrases

The first membrane-bound isozyme described was **carbonic anhydrase IV** (CA IV) (Whitney & Briggle, 1982) which is a glycosyl phospatidylinositol (GPI)-anchored protein expressed widely in various tissues. The *CA4* gene is located on chromosome 17 (Okuyama et al., 1992; 1993), and the molecular weight of the human CA IV protein is 35,000 Da (Zhu & Sly, 1990). The physiological role of CA IV is to facilitate the reversible hydration of carbon dioxide (CO_2) at the sites of a rapid flux of CO_2 and HCO_3^- across membranes (Sly & Hu, 1995).

CA IV is expressed on the luminal surface of pulmonary endothelial cells in lungs catalyzing the dehydration of bicarbonate to carbon dioxide (Fleming et al., 1993; Zhu & Sly 1990). On the brush border membrane of the proximal tubular cells and on the cells of thick ascending limbs of Henle, in kidney, CA IV facilitates bicarbonate reabsorption (Brown et al., 1990; Zhu & Sly 1990). CA IV on the apical surface of the epithelial cells in the colon and in distal small and large intestine participates in ion and fluid transport (Fleming et al., 1995). CA IV is localized also in human epididymis and ductus deferens of the male genitourinary tract (Ghandour et al., 1992), in the capillary endothelium of skeletal and heart muscle (Sender et al., 1994; Sender et al., 1998), and on the plasma face of endothelial cells of several capillary beds (Parkkila et al., 1996; Pastoreková et al., 1997; Schwartz, 2002;). Human CA IV has been also localized in the luminal plasma membrane of the gallbladder and bile duct epithelium (Parkkila et al., 1996), pancreas, salivary glands (Fujikawa-Adachi et al., 1999a), in the brain capillary endothelial cells (Ghandour et al., 1992), in erythrocyes (Wistrand et al., 1999) and choriocapillaris of the eye (Hageman et al., 1991).

Carbonic anhydrase IX (CA IX) is a 54,000/58,000 Da mass transmembrane glycoprotein with a signal peptide, proteoglycan-related sequence, transmembrane segment, complete CA domain in the middle of its large extracellular segment, and a short intracellular tail (Pastorekova & Zavada, 2004). CA IX was first found as a tumor-associated antigen, MN, in the normal gastric mucosa and human carcinomas (Pastorekova et al., 1992; Pastorek et al., 1994). CA IX is expressed at the basolateral

plasma membrane of epithelial cells and also in some cases in the nucleus. A human *CA9* gene has been mapped to chromosome 17 (Ivanov et al., 1998). The CA IX enzyme is expressed in the gastric epithelium (Pastorek et al., 1994), and the CA IX-positive cell types in the gastric mucosa were first defined by Pastoreková et al. (1997). CA IX expression has been observed also in the biliary epithelial cells (Parkkila & Parkkila, 1996; Pastoreková et al., 1997), and the presence of CA IX in the colonic enterocytes has been reported by Saarnio et al. (1998). After the discovery of the CA domain it was named as CA IX. The isozyme IX has restricted distribution (neoplastic cells) which could suggest its role in cell proliferation (also non-malignant) and transformation. A recent study on CA IX-deficient mice produced by targeted mutagenesis revealed that the enzyme deficiency results in a marked hyperplasia of mucus-producing cells in the gastric mucosa (Ortova Gut et al., 2002). These results suggest that CA IX is functionally implicated in gastric morphogenesis via the control of cell proliferation and differentiation.

Carbonic anhydrase XIV (CA XIV) is a transmembrane protein (Mori et al., 1999) with an amino-terminal signal sequence, a CA domain with high homology with other extracellular CAs, a transmembrane domain, and a short intracellular C-terminal tail. The *CA14* gene has been mapped to chromosome 1q21 (Fujikawa-Adachi et al., 1999c). Whittington et al. (2004) have suggested that the activity of CA XIV migth be higher than that of CA II. CA14 mRNA expression has been demonstrated in the human heart, brain, liver, spinal cord and skeletal muscle and a weak expression has been detected in the small intestine, colon, kidney, and urinary bladder by RNA dot blot analysis (Fujikawa-Adachi et al., 1999c). In the mouse kidney CA14 mRNA is expressed also in the apical and basolateral plasma membranes of the S1 and S2 segments of the proximal tubules, in the outer border of the inner stripe of the outer medulla and in the initial portion of the thin descending limb of Henle (Kaunisto et al., 2002). Parkkila et al. (2001) observed in their studies a strong expression of CA XIV in the neuronal membranes and axons in different parts of the human and mouse brain. This could suggest the role of CA XIV in modulation of excitatory synaptic transmission in the brain. A recent study has demonstrated an abundant expression of CA XIV at the plasma membrane of murine

hepatocytes (Parkkila et al., 2002) where it might regulate the pH and ion homeostasis between the bile canaliculi and hepatic sinusoids.

Most recently Hilvo et al. (2005) have described a novel member of -CAs called **CA XV**. At least eight species have genomic sequences encoding CA XV in which all the amino acid residues critical for CA activity are present. Apparently CA XV has become a non-processed pseudogene in humans and chimpanzees and studies with RT-PCR confirmed that humans do not express CA XV. In mice positive expression of CA XV mRNA can be seen in the kidney, brain, and testis. Based on a phylogenetic analysis mouse CA XV is closely related to CA IV, and CA XV also shares several structural properties with CA IV, i.e., it is a glycosylated, GPI-anchored membrane protein, and it binds CA inhibitor. Similar to mouse CA IV, the catalytic activity of CA XV is low. CA XV is probably the first member of the -CA gene family which is expressed in several species but not in humans and chimpanzees.

2.3. CARBONIC ANHYDRASE XII

2.3.1. General aspects

Human CA XII was originally cloned and characterized by two groups independently (Türeci et al., 1998; Ivanov et al., 1998), in both cases as a gene whose mRNA is greatly upregulated in renal cell carcinomas. The cDNA sequence predicted a 354-amino acid polypeptide with a molecular mass of 39,448 Da. The *CA12* gene has been mapped to chromosome 15q22 by fluorecence *in situ* hybridization (Türeci et al., 1998). CA XII is a one-pass, type I transmembrane protein with a 29-amino acid signal sequence (predicted cleavage between Gly-1 and Ser-1), 261-amino acid CA domain (homology domain), an additional short extracellular segment, a 26-amino acid hydrophobic transmembrane domain, and a 29-amino acid C-terminal cytoplasmic tail which contains two potential phosphorylation sites. CA XII protein sequence has a sequence identity of 30-42 % to other CAs (Türeci et al., 1998) and the extracellular domain contains three zinc-binding histidine residues (His-94, His-96 and His-119) which are present in the active sites of

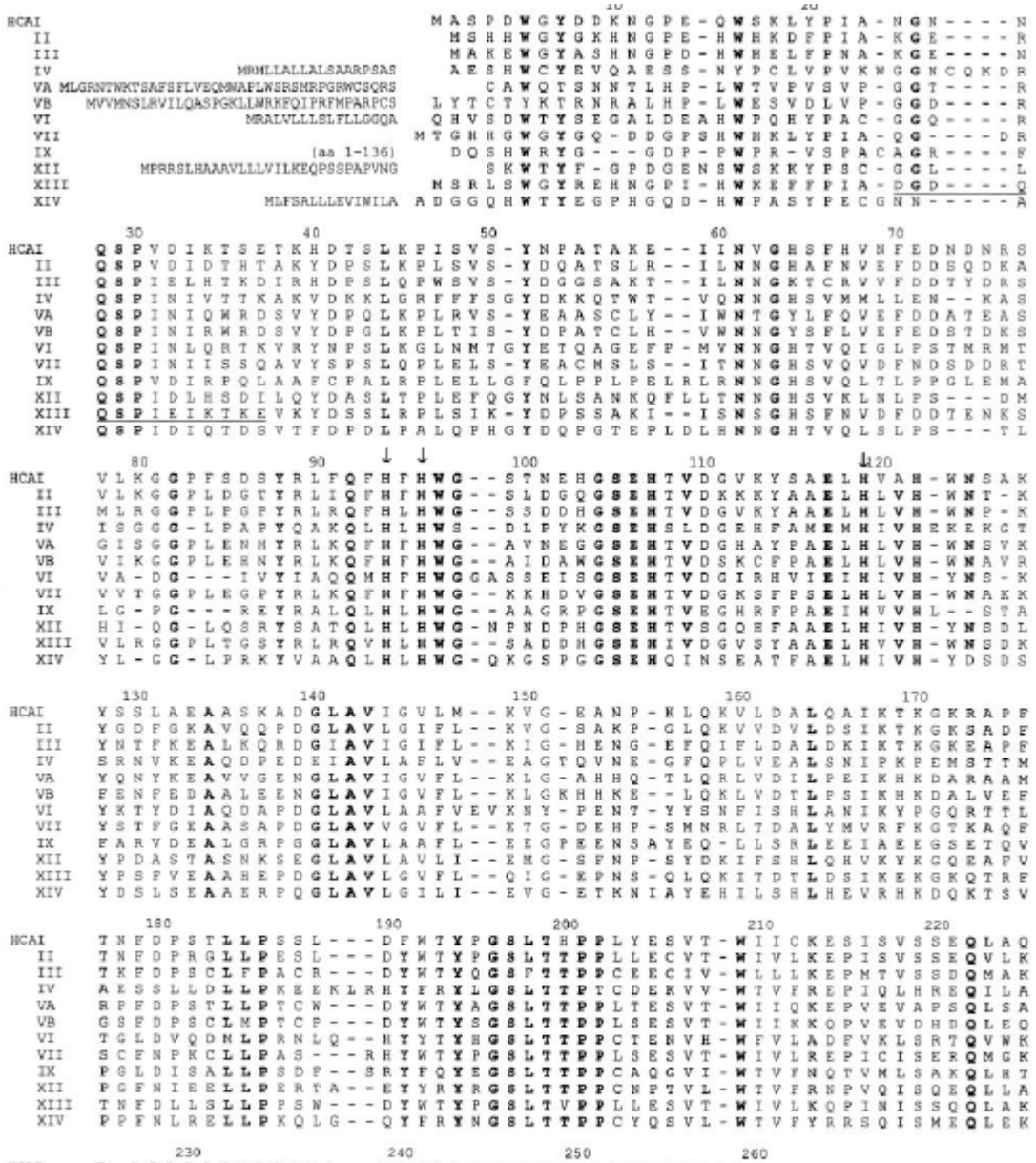

Figure 2.5. Amino acid sequence of human catalytic CAs. The aligned sequences correspond to numbering of amino acids in CA I (numbers above). The conserved residues are bold-faced, and arrows above His-94, His-96 and His-119 indicate Zn binding site residues. (Modified from Lehtonen et al., 2004)

catalytically active CAs (figure 2.5.). Histidine residue 64 is also conserved and it has been shown to contribute to the efficiency of high-activity CAs by serving as a proton shuttle between the zinc-bound water molecule and surrounding buffer molecules. The extracellular domain contains two potential sites for aspargine glycosylation and four cysteine residues (Türeci et al., 1998).

The crystal structure of a secretory form of human CA XII at 1.55-Å resolution has been described, and in its native state, CA XII appears as a dimer (Whittington et al., 2001). The activity of CA XII is moderate and is approximately same as the activity of CA I (Ulmasov et al., 2000). The *CA12* gene has been identified as a von Hippel-Lindau target gene, suggesting a potential role for CA XII in von Hippel-Lindau carcinogenesis (Ivanov et al., 1998). Recently, CA XII has been found to be a hypoxia-inducible gene (Ivanov et al., 2001; Wykoff et al., 2001; Watson et al., 2003), possibly explaining its upregulation in certain tumors. These recent reports suggest that CA XII may be an excellent marker for hypoxia in tumors.

2.3.2. Expression of CA XII in normal tissues

CA XII has a wide distribution pattern in normal tissues (Ivanov et al., 1998; Karhumaa et al., 2000; Kivelä et al., 2000; Parkkila et al., 2000; Türeci et al., 1998). Its mRNA is expressed in several normal human tissues such as aorta, bladder, brain, colon, esophagus, kidney, liver, lung, lymph node, mammary gland, ovary, prostate, pancreas, peripheral blood lymphocytes, rectum, stomach, skeletal muscle, skin, spleen, testis, trachea and uterus (Türeci et al., 1998; Ivanov et al., 1998; Kivelä et al., 2000). In the brain, *CA12* gene was found to be expressed only in the corpus striatum (caudate nucleus and putamen) (Ivanov et al., 1998; 2001).

Immunohistochemical studies have shown that CA XII protein is expressed in the human reproductive tissues (surface and glandular epithelial cells of the human endometrium, occasional epithelial cells in the uterine cervix, syncytiotrophoblasts of the placenta, epithelium of the efferent ducts, apical mitochonria-rich cells of the epididymal duct),

colon (basolateral plasma membrane of the epithelial cells), mesothelial cells, and kidney (renal proximal tubule, distal convoluted tubules, intercalacted cells of the collecting duct), sweat glands of the skin, epithelium of the breast, salivary glands (ductal cells, mucous cells), upper respiratory system (submucosal glands), nose (epithelial cells of Schneider's membrane), pancreas (acinar cells). Limited expression can be found also in the prostate, vas deferens, and transitional mucosa of the renal pelvis. In the gastrointestinal tract CA XII expression is limited to the surface epithelium of the large intestine. In the nervous system, CA XII immunoreactivity is found in the choroid plexus, in limited numbers of ganglion cells of the cortex, in the posterior lobe of the pituitary glands, and in the remnant of Rathke's pouch. (Ivanov et al., 2001; Karhumaa et al., 2000a, 2001; Kivelä et al., 2000; Parkkila et al., 2000).

CA XII is present in almost all epithelial cells of the efferent ducts and localized to the basolateral plasma membrane. It has been localized to the same cells as AQP1 in the excurrent tubule system which could suggest a role in ion transport and fluid reabsorption. The fluid leaving testis is modified and concentrated in the excurrent ducts by water reabsorption coupled to active transport of sodium and chloride ions. CA XII probably participates in these transport processes. Sodium and chloride ions are transported through the basolateral membrane via a Cl^-/HCO_3^- exchanger and co-transporter Na^+/HCO_3^- (NBC) (Jensen et al., 1999a;1999b). NBC probably needs CA to eliminate CO_3^{2-} gradients across the membrane, and CA might prevent the accumulation of HCO_3^- like in the renal proximal tubule (Müller_Berger et al., 1997). The apical mitochondria-rich cells of the epididymal duct, where the CA XII expression was also demonstrated, are involved in acidification of the epididymal fluid (Brown et al., 1992; Karhumaa et al., 2001; Martínez-García et al., 1995).

The staining with an antibody against a secreted form of human CA XII showed positive expression in the basolateral membranes of cells of the thick ascending limb, proximal and distal tubules and in the principal cells of the collecting ducts of the human kidney. The cellular distribution of CA XII has suggested an important role for this isozyme in

normal renal physiology (such as acidification of urine) and regulation of water homeostasis (Kyllönen et al., 2003; Nielsen et al., 1993; Parkkila et al., 2000).

2.3.3. CA XII in tumors

The expression of CA XII in multiple cancers has been shown to be in high level (Ivanov et al., 2001), even though its function in normal and malignant tissues is not yet exactly known. The CA XII overexpression has been shown in some human renal cancer cells. The renal tumour tissue had higher CA XII transcript expression levels than in the surrounding normal kidney tissue in 10 % of the patients with renal cell carcinoma (Türeci et al., 1998). The isozyme XII detected in renal cell carcinomas was shown by sequencing to be identical to that of a normal kidney. CA XII could acidify the immediate extracellular milieu surrounding the cancer cells in malignant tumors which could create a microenvironment leading to tumor growth and spread. The active site of the enzyme is located on the cell exterior, and this way it could regulate the extracellular pH in close proximity to the epithelium. The CA XII could be functionally coupled to an unidentified bicarbonate transporter to move bicarbonate across the basolateral membrane into the epithelial cells and to another transporter on the apical surface to secrete the bicarbonate ions. CA XII could also participate in a ligand-binding domain which is involved in transformation of tumor cells in a process that could be enhanced by the CA activity at the membrane of highly proliferating cells. If the CA activity at the membrane played a role in transformation, there could be a possibility to develop isozyme-specific CA inhibitors that could diminish the transforming potential of the membrane CA (McKiernan et al., 1997, Türeci et al., 1998).

The expression of CA XII is restricted to certain cell type and degree of differentiation within a given organ. CA XII shows membrane-associated expression in most oncocytomas and clear-cell carcinomas. CA XII is co-expressed with CA IX in neoplastic tissues such as mesotheliomas and choroid plexus tumors. Very high expression of CA XII has been demonstrated in low-grade ductal carcinoma and lobular carcinoma of the breast, and low-grade gliomas of the brain. CA XII expression is strong also in renal

clear-cell carcinomas, chromophobic cell carcinomas and oncocytic tumors (Ivanov et al., 2001; Parkkila et al., 2000). mRNA expression of CA XII has also been studied in cultured pancreatic tumor cell lines (Nishimori et al., 1999) and overexpression has been detected in the small-cell lung carcinoma cells. It has been reported also that most colorectal tumors seem to have abnormal CA XII expression in the deep parts of the adenomatous mucosa where the expression increased with the grade of dysplasia. The diffuse expression of CA XII in most malignant tumors correlates with their biological behavour (Kivelä et al., 2000). The expression of CA XII in gastric tumors is only slightly higher than in normal gastric mucosa (Leppilampi et al., 2003).

The carbonic anhydrase XII could serve as a biomarker for some malignant tumors (e.g. non-small cell lung carcinoma) and could be considered a potential target for novel therapeutic applications (Ivanov et al., 1998). CA XII has been demonstrated to be a good prognostic marker in invasive breast carcinoma patients (Watson et al, 2003).

2.3.4. Von Hippel-Lindau

Both CA IX and XII are functionally related to von Hippel-Lindau-mediated carcinogenesis and down-regulated by expression of the wild-type von Hippel-Lindau tumor suppressor protein (pVHL). Germline mutations in the *VHL* genes of humans cause a hereditary cancer syndrome, which is called the von Hippel-Lindau disease (Kondo & Kaelin, 2001).

The solid tumours develop in two stages. In the first stage the malignant cells grow into small tumours, and as soon as they face hypoxia the growth is ended. In the second stage, hypoxia causes severe changes in gene expression and leads to clonal selection within the tumour cell population, which is followed by angiogenesis and fundamental changes in energy metabolism (respiration is replaced by glycolysis) (Dang & Semenza, 1999; Hanahan & Folkman, 1996; Semenza, 2000; Stubbs et al., 2000; Warburg, 1930;). Consequently, the tumour microenvironment shows low oxygen tension, high hydrostatic pressure, and acidic extracellular pH (Helmlinger et al., 1997; Jain, 1999). Expression of

genes for angiogenesis, energy metabolism and transmembrane CAs is controlled by the hypoxia-inducible transcription factor, HIF-1, which integrates pathways regulating physiological responses to acute and chronic hypoxia (Bunn & Poyton, 1996; Gleadle & Ratcliffe, 1998; Gnarra et al., 1996; Hanahan & Folkman, 1996; Ivanov et al., 1998; Kaelin & Maher, 1998; Maxwell et al., 1999; Ohh & Kaelin, 1999; Semenza, 1999; 2000). The ubiquitinproteasome proteolysis system releases cellular proteins from a multiprotein ubiquitin (Ub) ligase complex, VBC (VHL/Elongin B/ElonginC). pVHL is an integral part of the complex, and targets for the proteolytic degradation are HIF-1α and

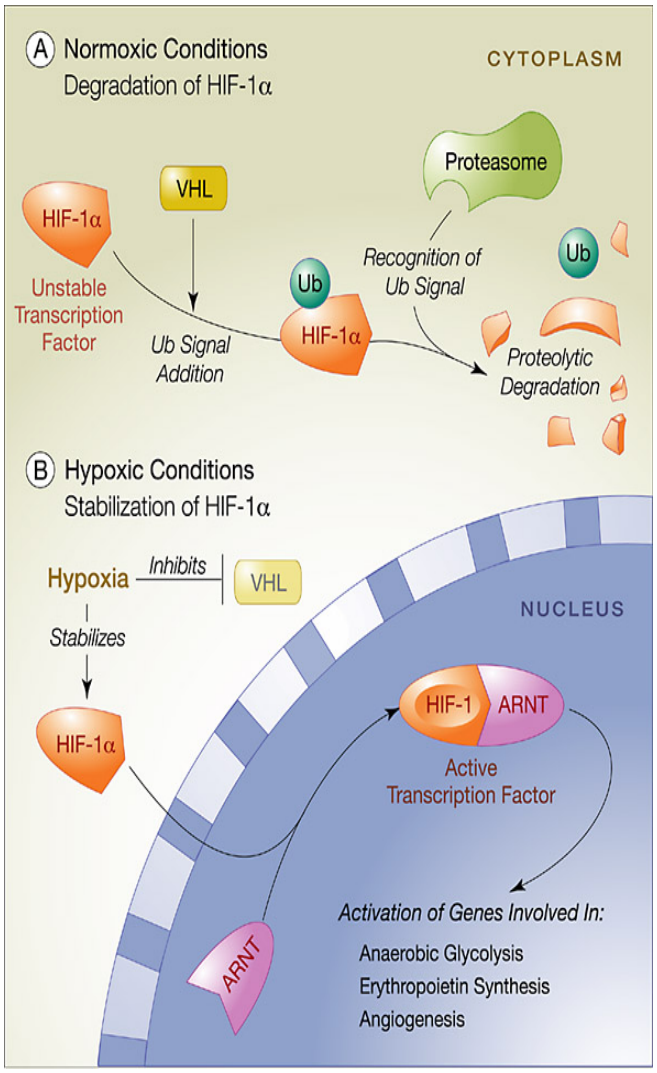

Figure 2.6. The hypoxia-inducible factor pathway of hypoxia control. As tumor masses expand, they outrun their oxygen supply requiring the synthesis of new capillaries, more red blood cells, and a switch to anaerobic glycolysis in order to survive and progress through what would otherwise be a lethal threat. Hypoxia-inducible factor (HIF-1α) is an unstable protein that fails to accumulate in cells except when they are exposed to a hypoxic environment. Through inhibitory signals that are processed by the von Hippel-Lindau (VHL) protein, HIF-1α is stabilized, accumulates, and interacts with ARNT (aryl hydrocarbon receptor nuclear translocator), its physiological partner, to form an active transcription factor. HIF-1α activates a number of cellular genes, including those for proteins that carry out anaerobic glycolysis, for erythropoietin (red blood cell production), and for vascular endothelial cell growth factor (VEGF) (Modified from Livingston & Shivdasani, 2001).

HIF-2α which both bind pVHL and degradate in normoxic conditions, not in hypoxia (Iwai et al., 1999; Maxwell et al., 1999; Tyers & Rottapel, 1999; Tyers & Willems,

1999). Therefore pVHL is the cause of the hypoxia-driven changes in gene expression in tumors. In VHL patients *CA12* gene is overexpressed in tumors because of the absence of pVHL (Ivanov et al., 1998). Ivanov et al. have shown in 2001 that hypoxia causes upregulation of *CA12* expression in tumor cell line expressiong a normal VHL message (figure 2.6.).

2.4. ACATALYTIC CARBONIC ANHYDRASES

The carbonic anhydrase related proteins, CA-RPs, are inactive isoforms in which one or more of the three critical zinc ion binding histidine residues in the active site have amino acid substitutions. Even though the function of these acatalytic isoforms is unknown, high sequence homologies between human and mouse cDNAs suggest that they have biologically important roles in higher animals. Acatalytic CA domain forms a ligand-binding domain for the two members of the receptor-type protein tyrosine phosphatase (RPTP) family (RPTP-β and RPTP-γ). They might also have an association with cancer since one of the RPTPs (RPTP-γ) could function as a tumor suppressor protein (Barnea et al., 1993; Nishimori, 2004; Wary et al., 1993).

The mammalian α-CA gene family includes at least three CA-RPs. **CA-RP VIII** has been identified in the Purkinje cells of the cerebellum (Kato, 1990), and it has the most highly conserved amino acid sequence of the α-CAs between the human and mouse homologues (~98%) (Skaggs et al., 1993). The amino acid sequence of CA-RP VIII is deduced with changes in the catalytic site residues which could explain the loss of the CA activity of this isoform. The CA-RP VIII is highly expressed in the murine brain (the granular and molecular layers of the cerebellum, the pial surface and in the large neurons throughout the cortical layer, the medial habenulae, neurons of the thalamus, strongly expressed in the hippocampal formation, specifically in the pyramidal cells of the Ammon's horn and in the granular cells of the dentate gyrus, and in the choroids plexus and cerebrospinal fluid-containing channels), liver, lung, heart, gut, and thymus (Lakkis et al., 1997; Ling et al., 1994, Skaggs et al., 1993). The CA-RP VIII is expressed also in the human testis, salivary glands, placenta and lungs (Ling et al., 1994; Skaggs et al., 1993). The

expression of murine CA-RP VIII mRNA is relatively high in the adult and fetal lung whereas it has not been found in the human adult lung tissue (Lakkis et al., 1997; Akisawa et al., 2003). The CA-RP VIII is strongly expressed in non-small cell lung carcinomas (at the invasion front). This suggests that the CA-RP VIII would be an oncofetal antigen and would have a role in non-small cell lung carcinomas. The wide distribution of its mRNA in the mouse brain could suggest a non-specific but generalized function for CA-RP VIII in the brain tissue.

2.5. CARBONIC ANHYDRASES IN NERVOUS SYSTEM

Over sixty years ago Ashby (1943) demonstrated CA activity in mammalian brain where it has various physiological functions such as fluid and ion compartmentation (Bourke & Kimelberg, 1975), the formation of cerebrospinal fluid (CSF) (Maren, 1967), seizure activity (Anderson et al., 1984), the respiratory response to carbon dioxide (Ridderstråle & Hanson, 1985) and the generation of bicarbonate for biosynthetic reactions (Tansey et al., 1988; Cammer, 1991). Giacobini (1961; 1962) reported the first suggestions about glia being the main site of CA expression in the central nervous system (CNS). His experimentations on CA activity in glial cells of rat brainstem were made by dissection of neurons and glial cell clumps. In 1964 Korhonen et al. showed that the areas rich in myelinated fibres and glial cells of the mouse brain have the highest CA activity. When the glial cells and neurons were separated by bulk isolation, the higher CA activity was detected in the glia than in neurons (Sinha & Rose, 1971; Nagata et al., 1974). Significant CA activity was also observed in the myelin isolated from rat, mouse, monkey, cat, and rabbit brains (Cammer et al., 1976; 1977; Sapirstein & Lees, 1978). In bulk-isolated cells, the oligodendrocytes seemed to have the highest activities whereas the neurons and astrocytes had only very low activities (Snyder et al. 1983).

Immunostaining of rodent and human brains and spinal cords with antisera against CA II showed positive signal in the oligodendrocytes (Roussel et al., 1979; Ghandour et al., 1979; 1980; Langley et al., 1980; Kumpulainen & Korhonen, 1982; Kumpulainen et al., 1983) and in myelin sheaths (Roussel et al., 1979; Kumpulainen & Korhonen, 1982).

Low levels of CA has been observed in the astrocytes in cultured cells (Kimelberg et al., 1982), in bulk isolated cells (Snyder et al., 1983), and in one immunohistochemical study on brain tissue sections (Roussel et al., 1979).

In other studies variable amounts of CA II have been observed also in the astrocytes (in the normal grey matter of the brain), in reactive astrocytes (in severely gliotic white matter of the jimpy mutant mouse and of rats with experimental autoimmune encephalomyelitis), in jimpy oligodendrocytes, and in reactive astrocytes, oligodendrocytes and neurons surrounding brain tumours and other types of neoplastic cells of these tumours (Borelli et al., 1982; Nakagawa et al., 1986; 1987; Cammer & Tansey, 1988; Ghandour & Skoff, 1988; Cammer, 1991; Jeffrey et al., 1991; Cammer & Zhang, 1992). An observation of Nogradi (1993) showed that the active brain macrophages express CA II and III, and the resting microglial cells express only CA III. The transformation of these microglial cells from a metabolically and/or immunologically more active form to less active (or vice versa), has been related to the immunoreactivity of CA II (Perry & Gordon, 1988; Ashwell, 1991; Nogradi, 1993).

CA activity has an important role in the production of CSF and in its regulation of pH and ionic constituents (Maren, 1967; Maren & Broder, 1970). The CA II and III activity has been observed in the cytoplasm and microvilli of the epithelial cells of the choroid plexus (Kumpulainen & Korhonen, 1982; Nogradi et al., 1993). The CA II activity and fatty acid synthase and acetyl-Coa carboxylase (first lipid synthesis enzymes) all exist in oligodendrocytes, which could suggest that CA II provides bicarbonate for the synthesis of fatty acids which form lipids to myelin (Tansey et al., 1988; Cammer, 1991). After the neurotoxic demyelination the CA activity is required for the compaction of myelin (Yanagisawa et al., 1990). The intracellular CA can be related to specialized sensory functions as it has been found in retinal neurons, in the sensory neurons of the ganglia in the peripheral nervous system, and in few CNS neurons (Neubauer, 1991). The localization of CA IV in the brain of adult rats and CA II-deficient mice is limited to the luminal surface of the capillary endothelial cells which could suggest an important role at the blood-brain barrier (Ghandour et al., 1992).

Table 2.2. Expression of CA isozymes in the brain.

Isozyme	Site of expression
II	oligodendrocytes astrocytes myelin choroid plexus neurons
III	choroid plexus microglial cells
IV	endothelial cells
V	astrocytes neurons
VII	choroid plexus pia neurons thalamus hippocampus cerebellum
XIV	neurons

Lakkis et al. (1997) demonstrated that there is CA VII mRNA expression in the pia, choroid plexus and neurons of the cortical layer, thalamus, and medial habenulae. A high expression can be seen in the pyramidal and granular cells of the hippocampus and in the cerebellum. *Car7* and *Car8* are transcribed to different degrees in the Purkinje cells; lower expression has been observed in the molecular and granular cell layers. Transcription signals of *Car7* and *Car8* are excluded from white matter regions.

The mitochondrial isoforms (CA VB) has been observed in astrocytes and neurons (Ghandour et al., 2000). This isozyme could have an important role in astrocytes in gluconeogenesis by providing bicarbonate ions for the pyruvate carboxylase expressed in those cells (Yu et al., 1983). Two novel roles for neuronal CA V have been suggested such as the regulation of the intramitochondrial calcium levels and the regulation of neuronal transmission by facilitating the bicarbonate ion-induced GABA responses (Ghandour et al., 2000). The finding of evidence that carboxylation of pyruvate to malate occurs in neurons and that it supports formation of transmitter glutamate (Hassel, 2001)

has suggested that the previous predictions were not the final word in the neuronal metabolism of pyruvate, and that CA V might serve a number of different physiological processes in neurons.

Parkkila et al. (2001) demonstrated that CA XIV, a membrane-bound isozyme, is highly expressed in some neurons, highest expressed on large neuronal bodies and axons in the anterolateral part of the pons and medulla oblongata, in the hippocampus, corpus callosum, cerebellar white matter and peduncles, pyramidal tract, and choroid plexus.. The localization of CA XIV in neurons may have an important role in the production of alkaline shift linked to the neuronal signal transduction.

2.5.1. Formation of cerebro-spinal fluid (CSF)

The cerebro-spinal fluid (CSF) provides a protective buoyancy for the brain which effectively makes the weigth of the brain $1/30^{th}$ of its actual weigth. The CSF also provides maintenance of the chemical environment of the central nervous system. The way in which the metabolites are removed is a one-way flow of CSF from ventricular system, around the spinal cord, into the subarachnoid space and into the venous sinuses. Nowhere in the body there's more need for homeostasis than in the brain. The mechanism for maintenance of this barrier function lies in the capillary network supplying blood to the brain. The blood-brain barrier (BBB) protects the brain against surging fluctuations in ion concentrations of the plasma (Davson et al., 1987).

The concept of blood-brain barrier shows that the BBB is located in endothelial cells of capillaries of the brain. These endothelial cells in the brain are different to those in peripheral tissues. Brain endothelial cells are joined by tight junctions of high electrical resistance. In brain endothelial cells there's no movement like in peripheral endothelial cells. Brain capillaries are in contact with foot processes of astrocytes which separate the capillaries from the neurons. The BBB is both a physical barrier and a system of cellular transport mechanisms. It maintains homeostasis by restricting the entrances of potentially harmful chemicals from the blood, and by allowing the entrance of essential nutrients

(Davson et al., 1987). Lipid soluble molecules (such as ethanol) are able to penetrate trough the BBB relatively easily via the lipid membranes of the cells. Water soluble molecules (such as sodium) are unable to transverse the barrier without the use of specialized carrier-mediated transport mechanism (figure 2.9.). There are some areas of the brain that do not have a blood-brain barrier. The BBB is absent in the pituitary because it allows products to pass into the circulation. Another are is a chemoreceptive area which allowa transcellular transport for the water balance and other homeostatic functions (Laterra et al., 1991; Schmidley & Maas, 1990).

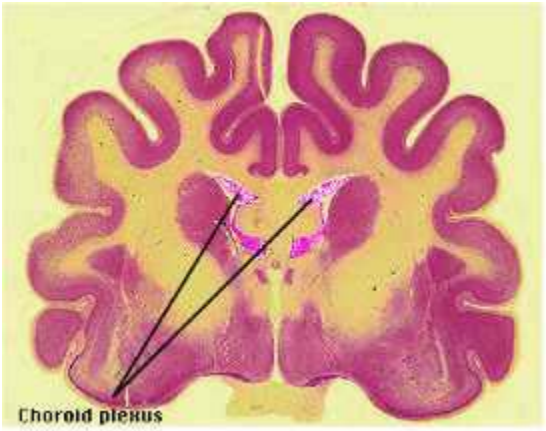

Figure 2.7. The picture on the left is a sagital section of a human brain showing the location of the choroid plexus in the ventricular system (Modified from http://cal.vet.upenn.edu/neuro/server/slides/ns_075-Ch.jpg).

The ependymal cells that line the ventricles of the brain fuse with the pia mater to form the choroid plexus (figure 2.7.). The choroid plexusas are branched structures made up of numerous villi. They provide a large surface area for the secretion of CSF. The choroid plexus has a rich blood supply. Capillaries in the choroid plexus are highly specialized for their function. They are fenestrated and provide little resistance to the movement of small molecules. The epithelial cells of the choroid plexus are linked by junctional complexes, thus the epithelium forms the blood-CSF barrier (Johanson et al., 1995).

CSF is secreted by the epithelial cells of the choroid plexuses which are polarised so that the properties of their apical membrane (facing ventricle) differ from those of the basolateral membrane (facing blood). Both of these membranes have a greatly expanded area, so that the total area available for transport is similar to that of the BBB. CSF secretion involves the transport of ions (Na^+, Cl^- and HCO_3^-) across the epithelium from

blood to CSF (figure 2.8.). This movement of ions creates an osmotic gradient that drives the unidireccional transport of ions across an epithelium. Secretion can occur because of the polarised distribution of specific ion transporters in the apical or basolateral membrane of the epithelial cells (Siegel et al., 1999).

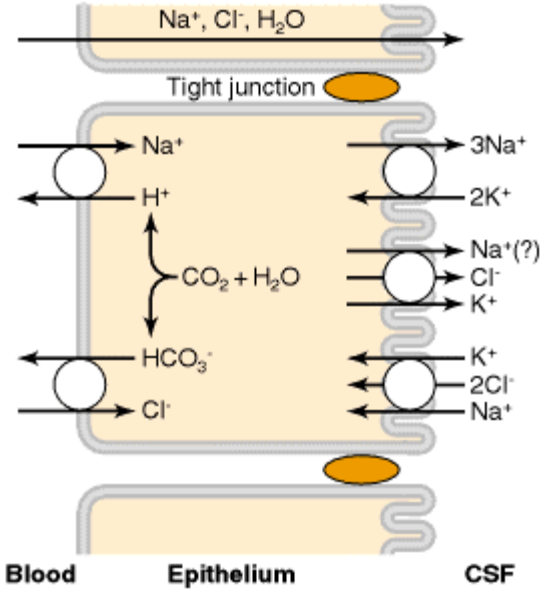

Figure 2.8. Model of ion transport at the choroid plexus epithelium. Net transport of Na^+ and Cl^- across the epithelium results in the secretion of CSF. Cl^- efflux from the epithelium to CSF is mediated by a cotransporter. It is uncertain whether that transporter is of the $Na^+/K^+/2Cl^-$ or K^+/Cl^- form. The generation of H^+ and HCO^-_3 by carbonic anhydrase is important in the secretion of CSF (Modified from Siegel et al., 1999).

The choroid plexus consist of highly vascularized masses of pia mater tissue that dip into pockets formed by ependymal cells (figure 2.9.). The preponderance of choroid plexus is distributed throughout the fourth ventricle near the base of the brain and in the lateral ventricles inside the right and left cerebral hemispheres. The cells of the choroidal epithelium are modified and have epithelial characteristics. These ependymal cells have microvilli on the CSF side, basolateral interdigitations, and abundant mitochondria. The ependymal cells, which line the ventricles, form a continuous sheet around the choroid plexus. While the capillaries of the choroid plexus are fenestrated, non-continuous and have gaps between the capillary endothelial cells allowing the free-movement of small molecules, the adjacent choroidal epithelial cells form tight junctions preventing most macromolecules from effectively passing into the CSF from the blood (Brightman, 1968; Redzic & Segal, 2004).

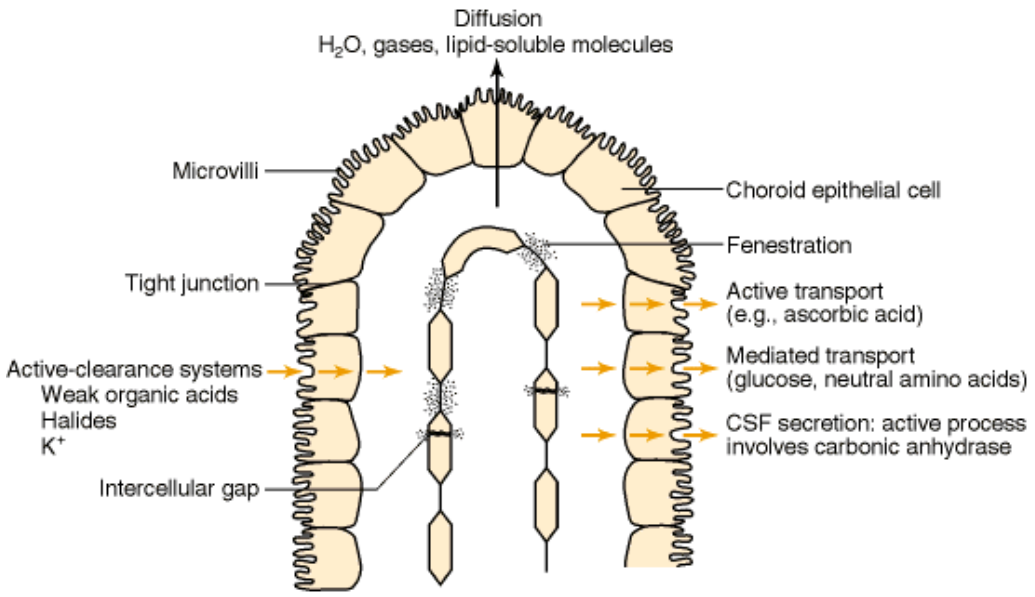

Figure 2.9. Blood—CSF barrier. The blood—CSF barrier is at the choroid plexus epithelial cells, which are joined together by tight junctions.The capillaries in the choroid plexus differ from those of the brain in that there is free movement of molecules across the endothelial cell through fenestrations and intercellular gaps. Microvilli are present on the CSF-facing surface and they increase the surface area of the apical membrane (Modified from Siegel et al., 1999).

2.6. CARBONIC ANHYDRASE INHIBITORS

Regulation of the acid-base balance is a physiological process, which involves a number of proteins such as ion transport proteins, plasma membrane receptors and their ligands, and CAs. Different CA isozymes have an important role in ion and water transport and some isozymes may physically interact with various ion transporters (Casey et al., 2004).

Carbonic anhydrase inhibitors (CAIs) can be classified into two groups: the metal-complexing anions and the unsubstituted sulfonamides. Sulfonamides are the most important CAIs because they bind in a tetrahedral geometry of the zinc ion and forms a network of hydrogen bonds involving many amino acids as well as the metal ion (Supuran, 2004). The major applications of CA inhibitors are used in opthalmology. Acetazolamide, methazolamide, ethoxzolamide and dichlorophenamide are systemic antiglaucoma drugs, which inhibit CA II and CA IV present in the ciliary processes of eye. The inhibition of CAs prevents the sympthoms of glaucoma by reducing the

secretion of aqueous humor and HCO_3^- and lowering the intraocular pressure (Mincione et al., 2004).

Acetazolamide has been observed to inhibit both Na^+ and Cl^- absorption in human intestines (Turnberg et al., 1970a; 1970b), and this proposes that most of the absorption must be mediated by electroneutral Na^+-H^+ and Cl^--HCO_3^- exchange processes. Because water absorption follows ion movements, are CAs probably also implicated in water absorption. Abundantly expressed in the non-goblet epithelial cells of the mammalian colon, CA I and II are probably key players in this physiological process (Lönnerholm et al., 1985; Parkkila et al., 1994). The luminal content of the colon is alkalized by bicarbonate secretion, which depends on apical Cl^--HCO_3^- exchange (Feldman & Stephenson, 1990) and it acidifies the luminal content by active proton secretion (Suzuki & Kaneko, 1987). This proton secretion can facilitate non-ionic fatty acid uptake by promoting apical Na^+-H^+ exchange (Sellin & DeSoignie, 1990) or a proton ATPase pump (Gustin & Goodman, 1981).

There may be several CA isozymes involved in the regulation of the acid-base balance in the alimentary tract. The clinical applications of CA inhibitors have been limited in the gastrointestinal tract so far. One of the most attractive applications is that CA inhibitors could be useful for the therapy of peptic ulcer. An early approach to attack the machinery of the acid-producing cell by acetazolamide was discovered by Baron (2000). Davenport suggested in 1939 that CA might be essential for acid production, so an inhibitor of this enzyme would inhibit gastric acid secretion. A brief acid inhibition by acetazolamide was demonstrated and concluded that its action was too brief to be therapeutically useful (Janowitz et al., 1952; 1957). But later studies showed that acetazolamide might be effective in the treatment of gastric ulcer (Puscas et al., 1989; Erdei et al., 1990). Acetazolamide has never generally been approved for the treatment of gastric ulcer because it has many unfavourable side effects and documentation of its efficacy has been insufficient.

The CA activity in renal acidification has been studied using CA inhibitors, although they have not been very useful in the therapy of renal diseases (Supuran & Scozzafava, 2000). Acetazolamide, methazolamide, ethoxzolamide and dichlorophenamide can be used for the treatment of edema induced by drugs or congestive heart failure (Supuran & Scozzafava, 2000) but they can cause numerous undesired side effects, such as metabolic acidosis, nephrolithiasis, CNS symptoms and allergic reactions (Tawil et al., 1993; Supuran et al., 2001). The acute response to CA inhibition is an increase in the excretion of bicarbonate, sodium and potassium, an increase in urinary flow, and titratable acid (Bagnis et al., 2001) and the loss of bicarbonate and sodium is considered self-limited on continued administration of the inhibitor, probably because the initial acidosis resulting from bicarbonate loss activates bicarbonate reabsorption via CA-independent mechanisms. Chronic CA inhibition stimulates morphologic changes in the collecting ducts (Bagnis et al., 2001) and therefore, CA activity could play an important role in determining the differentiated phenotype of renal epithelium.

2.6.1. CA inhibitors in the nervous system

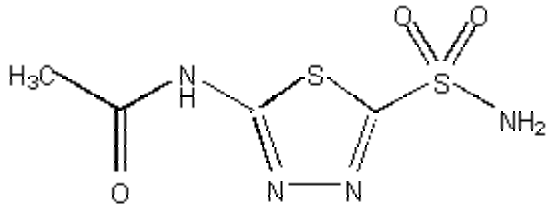

Figure 2.10. Acetazolamide.

CA inhibitors have profound effects on the function of the central nervous system (CNS). **Acetazolamide** (figure 2.10.) can reduce CSF (cerebro-spinal-fluid) production by about 50 per cent (Maren, 1972; McCarthy & Reed, 1974), the concentration of carbon dioxide in brain tissues increases and probability of seizures decreases. It is also known that it dilates intracranial vessels (Maren, 1967; Hauge et al., 1983) which increases cerebral blood volume (CBV). This increase in CBV reflects an intrinsic volume load to the intracranial cavity and with normal CSF circulation and absorption it does not elevate significantly the intracranial pressure (Parkkila et al., 2004).

Pseudotumor cerebri is a syndrome where acetazolamide is considered a drug of choice (Shin & Balcer, 2002). In pseudotumor cerebri the patient has elevated intracranial pressure, normal cerebral anatomy, normal CSF fluid composition, and signs and symptoms of intracranial pressure. Acetazolamide causes long-lasting control of transient visual obscuration, headache, and diplopia, which are manifestations of intracranial hypertension.

An acute mountain sickness can cause high altitude cerebral edema which can occur at heights above 4500 meters. The clinical features include headache, impairment of consciousness and a variety of neurological signs (Clarke, 1988). Acetazolamide is an important drug for the management of high-altitude illness. Also dexamethasone and oxygen can be managed as well as use of portable hyperbaric chamber (Hackett & Roach, 2001).

Acetazolamide has been used for the treatment of epilepsy (Reiss & Oles, 1996) primarily as a combination therapy with other antiepileptic medications especially in refractory epilepsy (Reiss & Oles, 1996; Katayama et al., 2002). Acetazolamide may be useful in partial, myoclonic, absence, and primary generalized tonic-clonic seizures uncontrolled by other marketed agents.

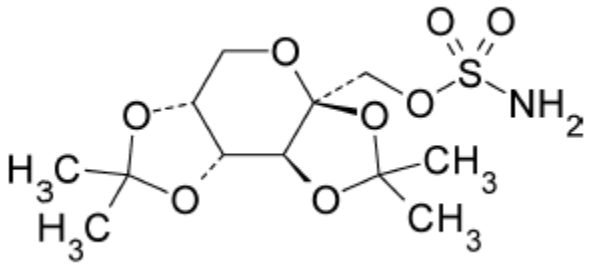

Figure 2.11. Topiramate.

Another considered CA inhibitor is **Topiramate**. It's a sulfamate fructo-pyranose derivative which is currently available for the treatment of partial onset and generalized epileptic seizures in adults and children (Bialer et al., 1999). Topiramate has a different

structure compared to the other commonly used anti-epileptic drugs (Perucca, 1997) and it shares the ability to inhibit CA activity with other sulfamate or sulfonamide derivatives (figure 2.11.). Topiramate seems to inhibit more potentially CA II and CA IV than CA I, CA III, and CA VI (Dodgson et al., 2000) and it has been demonstrated to be a strong CA inhibitor of human CA II isoenzyme (Supuran & Scozzafava, 2000). Due to some studies it has been suggested that inhibition of CA would not be the main mechanism responsible for its activity (Perucca, 1997; Stringer, 2000), but the anticonvulsant actions of topiramate would involve several mechanisms such as enhancement of GABAergic transmission and inhibitory action on neuronal sodium currents and that topiramate would inhibit excitatory transmission by antagonizing some types of glutamate receptors.

2.7. KAINIC ACID INDUCED STATUS EPILEPTICUS

2.7.1. Status epilepticus

Status epilepticus means a state where the seizures last unusually long (>10 minutes) or they repeat with short breaks without that the patient has time to recover from the last seizure. Seizures result from paroxysmal and excessive electrical neuronal discharges in the brain which have a variety of clinical manifestations. The term epilepsy is usually restricted to those cases with a tendency for recurrent seizures and the identification of a seizure as a symptom and not a disease diagnosis is important. There are several factors which can cause these seizures such as lack of sleep, infections or forget to administrate the medication. Often the underlying disease is epilepsy, but at other times it may be a non-epileptic disorder such as brain tumor, an infection of central nervous system, alcohol, drugs or imbalances of fluid homeostasis that cause a symptom resembling an epileptic seizure (Hauser et al., 1990).

Two sets of changes can determine the epileptogenic properties of neuronal tissues. Abnormal neuronal excitability occurs as a result of disruption of the depolarization and repolarization mechanisms of the cell (excitability of neuronal tissue). Aberrant neuronal networks that develop abnormal synchronization of a group of neurons can result in the

development and propagation of an epileptic seizure (synchronization of neuronal tissue) (Engel, 1989).

A hyperexcitability of neurons that results in random firing of cells may not lead to propagation of an epileptic seizure. Both normal and abnormal patterns of behaviour require a certain degree of synchronization of firing in a population of neurons. Epileptic seizures originate in a setting of both altered excitability and altered synchronization of neurons. The excitability of individual neurons is affected by cell membrane properties and the microenvironment of the neuron, structural features of neuronal elements, intracellular processes and interneuronal connections (Morgan et al., 1987).

The membrane properties and microenvironment of neurons, which maintain potential differences of electrical charge, are determined by selective ion permeability and ionic pumps. Excitatory neurotransmitters act by opening Na^+ or Ca^{2+} channels, whereas inhibitory neurotransmitters open K^+ or Cl^- channels. The mechanism of action of certain anticonvulsant medications is by Na^+ or Ca^{2+} channel blockade, which can prevent repetitive neuronal firing. Extracellular ionic concentrations also can contribute to neuronal excitability. For example, an increase in extracellular K^+ concentrations (such as in rapid neuronal firing, which is mainly responsible for K^+ reuptake) causes membrane depolarization (Morgan et al., 1987).

Various intracellular processes are controlled by genetic information. Neuronal excitability can be preprogrammed by DNA-controlled effects on cell structure, energy metabolism, receptor functions, transmitter release, and ionic channels. The mechanisms that induce these changes, either phasic or long-term, appear to be linked to ionic currents, especially Ca^{2+} influx. Intracellular Ca^{2+} mediates changes in membrane proteins to initiate transmitter release and ion channel opening. It also activates enzymes to allow neurons to cover or uncover receptor sites that alter neuronal sensitivity. Various plastic or persistent changes in excitability can result by influencing the expression of genetic information through Ca^{2+} influx. This may occur by selectively inducing genes to synthesize a protein for a specific reason. One example is the induction of the c-fos gene to produce c-fos protein in neurons involved in an epileptic seizure by the administration

of pentylenetetrazol. The exact effects of this coupling are not known, but it provides a means to study the effects of neuronal excitation on cell growth and differentiation as a model for epilepsy, learning, and memory (Morgan et al., 1987).

In regard to the structural features of neuronal elements in relation to epilepsy, the two primary regions of the brain that are involved in epilepsy are the cerebral neocortex and the hippocampus.

In the neocortex, excitatory synapses are made primarily on the dendritic spines and shaft, and the release of neurotransmitters at these sites gives rise to excitatory postsynaptic potentials. The inhibitory synapses are more prominent on the soma or proximal dendrites, and give rise to inhibitory postsynaptic potentials. The placement of these synapses effectively prevents distal excitatory events from reaching the axon hillock. Alterations of neuronal morphology could enhance excitability with either an actual increase in the number of excitatory synapses or a decrease in the number of inhibitory synapses. Such alterations could consist of reduced dendritic branching with excitatory synapses placed closer to the axon hillock, or loss of spines, allowing more excitatory synapses to occur directly on the shaft. Lesions in the neuronal cell body or tracts lead to degeneration of the axon terminal, and a new terminal may sprout to make contact with the vacated postsynaptic membrane, which may in turn lead to an increase in the excitatory potential of the neuron. Ca^{2+} currents that occur predominantly at the dendrites cause a high-amplitude prolonged depolarization that can evoke a rapid train of Na^{+} action potentials (burst-firing of Na^{+}), which is followed by a prolonged after hyperpolarization. These discharges are believed to contribute to the paroxysmal depolarization shifts and after hyperpolarization in experimental epileptic foci (Messenheimer et al., 1979).

Neurons are influenced by synaptic and nonsynaptic interconnections. Neurochemical transmission between neurons involves a number of steps that can be selectively altered to affect neuronal excitability. These steps result in the release of neurotransmitter into the synaptic cleft and the postsynaptic membrane, resulting in excitatory or inhibitory postsynaptic potentials via Ca^{2+} and other second messengers. The transmitters are

deactivated by enzymes. The primary excitatory neurotransmitters in the central nervous system are the amino acids glutamate and aspartate. The primary inhibitory neurotransmitters in the central nervous system are gamma-aminobutyric acid (GABA) and glycine. Neurotransmitters and neuromodulators exert their effects by acting on receptors. Specific properties of receptors have been identified on the basis of the effects of certain agonist and antagonist agents, some of which are anticonvulsant drugs. $GABA_A$ receptor drugs (activate Cl^-) appear more effective as anticonvulsants than $GABA_B$ receptor agents (activate K^+). The $GABA_A$ receptor is of primary importance in absence epilepsy due to its role in the synchronization and desynchronization of thalamocortical pathways. The oscillatory and burst-firing of these circuits is attributed to neurons in the reticular nucleus of the thalamus and leads to synchronization and desynchronization of the electroencephalogram (EEG). Alterations of this mechanism produce absence seizures. Kainic acid, quisqualic acid, and N-methyl-D-aspartate (NMDA) are excitatory amino acid analogs used to define the classes of receptors responsive to glutamate and aspartate. NMDA antagonists are one potential mechanism for some of the anticonvulsants. Two hypotheses are associated with cortical dysplasia, a frequent cause of medically intractable focal epilepsy. The first suggests that epileptogenesis results from a change in the synaptic properties of interneurons. The second suggests abnormal intrinsic properties in the neurons, such as a mutation in the ion channel (Matsumoto & Aimone-Marsan C, 1964).

2.7.2. Kainic acid induced status epilepticus

Kainic acid induced status epilepticus produces damage in specific brain regions, and the most vulnerable areas are amygdaloid complex, entorhinal cortex, hippocampus, and piriform. The CA3 and CA1 regions of the hippocampus are most affected (Sperk, 1994). The fragmentation of the genomic DNA of dying neurons into internucleosomal size pieces by the kainic acid induced neuronal death is considered to be a hallmark of apoptosis. In some cases these apoptotic cells require specific novel proteins newly synthesized. Blocking this protein synthesis with cycloheximide prevents neuronal death caused by kainic acid induced seizures (Schreiber et al., 1993).The transcription of so-

called suicide genes can be regulated by immediate-early genes (IEGs) (Estus et al., 1994; Ham et al., 1995). This IEG expression continues for several days in vulnerable brains areas after a kainic acid induced status epilepticus (Popovici et al., 1990; Dragunow et al., 1993; Schreiber et al., 1993; Smeyne et al., 1993; Taniguchi et al., 1994).

Adult male rats are provoked a kainic acid induced status epilepticus by injecting them subcutanously with kainic acid. After the injection, first rats show behaviour such as staring and movement arrest and thereafter wet dog shakes eventually leading to unilateral limb and/or facial clonus. After 1 h from the injection, rats have the first generalized tonic-clonic seizure with bilateral limb clonus, which is considered as zero time point. Behavioural seizure activity slowly changes continuously during several hours.

3. AIMS OF THE RESEARCH

This study had two specific goals. The first one was to investigate CA XII mRNA and protein expression in mouse tissues. The mRNA expression was studied by a polymerase chain reaction (PCR) method, and the protein production was studied by an immunohistochemical method. The second goal was to specify the distribution and expression of CA XII in rat brain with a kainic acid induced status epilepticus model. This was done by *in situ* hybridization and northern blot methods.

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4. METHODS

4.1. EXPRESSION OF CA XII IN MOUSE TISSUES

4.1.1. Immunohistochemistry

4.1.1.1. Animal treatments and tissue preparations

Tissue samples of stomach, duodenum, jejunum, ileum, colon, rectum, liver, psoas muscle, heart, brain and testis were obtained for immunohistochemistry from two adult mice (Balb/c). These procedures were approved by the institutional animal care committee (University of Tampere). The specimens were fixed in Carnoy's fluid [absolute ethanol (Primalco) + chloroform (Merck) + glacial acetic acid (Merck) in 6:3:1 ratio] for 18 h at 4°C. Thereafter the samples were dehydrated at 4°C in absolute ethanol for 30 min, in ethanol + chloroform (1:1) for 15 min, and in chloroform for 30 min, and finally embedded in paraffin in a vacuum oven at 58 ºC. Sections of 4-µm were cut and placed on gelatin-coated microscope slides (SuperFrost, Menzel-Gläser, Germany). The samples were fixed on to the microscope slides over night at 37 ºC.

4.1.1.2. Production of polyclonal rabbit antibody

The production of polyclonal rabbit antibody raised against the recombinant mouse CA XII has been described by Kyllönen et al. (2003). The specificity of the antibody was confirmed by Western blotting.

4.1.1.3. Immunohistochemical method

The tissue sections were immunostained by the biotin-streptavidin complex method to locate the CA XII. The sections were deparafinated by rinsing them in decending alcohol series (3X xylene substitute, 2X absolute ethanol, 2X 94% ethanol, 1X 70% ethanol, H_2O; à 5 min). Thereafter the sections were treated with 3% H_2O_2 in phosphate-buffered saline (PBS) for 5 min and rinsed twice in PBS (à 5 min). The pre-treatment of the

sections was made by undiluted cow colostral whey (Biotop Oy, Oulu, Finland) for 30 min and then rinsing them in PBS. Then the sections were incubated in anti-mouse CA

1. Removal of paraffin	
decending alcohol series	
3 x xylene substitute	á 5 min
2 x absolute ethanol	á 5 min
2 x 94 % ethanol	á 5 min
1 x 70 % ethanol	á 5 min
1 x diluted water	á 5 min
2. Treatment with peroxide	
3 % H_2O_2 in 1 x PBS	5 min
2 x rinsing in 1 x PBS	á 5 min
3. Pre-treatment	
undiluted cow colostral whey	30 min
1 x dipping in 1 x PBS	5 s
4. Treatment with primary antibody	
anti-mouse serum CA XII diluted (1:100) in 1% BSA-PBS	1 h
3 x rinsing in 1 x PBS	á 10 min
5. Treatment with secundary antibody	
biotinylated goat anti-rabbit IgG diluted (1:300) in 1% BSA-PBS	1 h
3 x rinsing in 1 x PBS	á 10 min
6. Enzyme conjugate	
peroxidase-conjugated streptavidin diluted (1:750) in 1 x PBS	30 min
4 x rinsing in 1 x PBS	á 5 min
7. Staining	
9 mg DAB in 15 ml PBS + 5 ul 30%H_2O_2	1 min
8. Diluted water and decending alcohol series	
9. Mounting with Neo-mount	

Figure 4.1. Biotin-streptavidin complex method.

XII serum (1:100 in 1% bovine serum albumin (BSA)-PBS) for 1 h. Thereafter they were incubated for 1 h with biotinylated goat anti-rabbit IgG (Zymed Laboratories, South San Francisco, CA) diluted 1:300 in 1% BSA-PBS and for 30 min with peroxidase-conjugated streptavidin (Zymed Laboratories). The staining was made by incubating the sections in DAB solution containing 9 mg 3,3'-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO) in 15 ml PBS + 5 µl 30% H_2O_2 for 1 min. The sections were washed 3 times for 10 min in PBS after incubating in primary and secondary antibodies, and 4 times for 5 min in PBS after enzyme conjugate incubation. In the end of the staining all the sections were carried through an alcohol series (1X H_2O, 1X 70% ethanol, 2X 94% ethanol, 2X absolute ethanol, 3X xylene substitute). All the incubations and washings were carried out at room temperature in a humid champer. The stained sections were mounted with Neo-mount (Merck) and evaporated over night. Next day the sections were examined and photographed with Zeiss Axioskop 40 microscope (Carl Zeiss, Göttingen, Germany) (figure 4.1.).

4.1.2. PCR method

4.1.2.1. cDNA preparations

The expression of mouse CA XII mRNA was examined using a cDNA kit (MTC™ panel I) purchased from BD Biosciences (Palo Alto, CA). Mouse MTC™ panel I contained normalized (beta-actin, G3PDH, phospholipase A2, ribosomal protein S29), first strand cDNA preparations (1 ng/µl) produced from total poly A+ RNAs isolated from the tissues of mouse (BALB/c, Webster, Swiss Webster/NIH) heart, brain (whole), spleen, lung, liver, skeletal muscle, kidney, testis, 7-, 11-, 15- and 17-day embryos (table 4.1.). Panel cDNAs ranged in size from 0.1 to at least 6 kb and were virtually free of genomic DNA. The DNAse used to treat RNA samples was completely removed before the first-strand cDNA was synthesized. The cDNAs included in the MTC™ panel I were used as templates for polymerase chain reaction (PCR) using *CA12* gene specific primers. G3PDH (glyceraldehydes 3-phosphate dehydrogenase) PCR primers and control cDNA were included for positive control reactions.

Table 4.1. Tissue samples used in PCR method.

Tissue	Source
heart	pooled from 200 male/female BALB/c mice, ages 8-12 weeks
brain	pooled from 200 male/female BALB/c mice, ages 8-12 weeks
spleen	pooled from 200 male/female BALB/c mice, ages 8-12 weeks
lung	pooled from 200 male/female BALB/c mice, ages 8-12 weeks
liver	pooled from 200 male/female BALB/c mice, ages 8-12 weeks
skeletal muscle	pooled from 200 male BALB/c mice, ages 8-12 weeks
kidney	pooled from 200 male BALB/c mice, ages 8-12 weeks
testis	pooled from 200 BALB/c mice, ages 8-12 weeks
7-day embryo	pooled from 200 male/female Webster mice
11-day embryo	pooled from 200 male/female Webster mice
15-day embryo	pooled from 200 unknown Webster mice
17-day embryo	pooled from 200 male/female Swiss Webster/NIH mice

4.1.2.2. PCR method

The expression of mouse CA XII mRNA was examined by a cDNA kit (MTC™ panel I) which was used a template for polymerase chain reaction (PCR) using *CA12* gene-specific primers. Two primers for amplifying CA XII cDNA were designed based on the published mouse CA XII sequence in Genbank (accession numbers BC033432 and AK052639): forward 5'-TGGTGATCCTTAAGAAGCA-3' and reverse 5'-GCACAGGGTTTCGGAAAACT-3', which generated a 692-bp amplification product.

Table 4.2. The PCR master mix.

Reagent	Amount per reaction (µl)
PCR-grade deionized H2O	36
10x PCR reaction buffer	5
50x dNTP mix (10 mM each)	1
TITANIUM Taq DNA Polymerase (50x)	1
Total volume	43

Sigma Genosys (Cambridgeshire, UK) produced the primers. The quality of the cDNA preparations were monitored by primers for glyceraldehyde 3-phosphate dehydrogenase (G3PDH, BD Biosciences). Five nanograms (1 ng/ µl; 5 µl) of each cDNA sample were

used as a template for PCR reactions (PCR master mix and PCR set-up, table 4.2. and 4.3.).

Table 4.3. PCR set-up.

Component	Positive control (µl)	Negative control (µl)	G3PDH controls (µl)	*CA12* gene specific primers
PCR master mix	**43**	**43**	**43**	**43**
deionized H_2O	**-**	**5**	**-**	**-**
G3PDH primers	**2**	**2**	**2**	**-**
5' (upstream, 10 µM)	**-**	**-**	**-**	**1**
3' (downstream, 10 µM)	**-**	**-**	**-**	**1**
control DNA	**5**	**-**	**-**	**-**
cDNA panel sample	**-**	**-**	**5**	**5**
total volume	**50**	**50**	**50**	**50**

The PCR reaction was carried out on a thermal cycler (Gene Amp PCR system 9700: Applied Biosystems; Foster City, CA). The PCR cycling protocol consisted of denaturation at 94ºC for 1 min, followed by 33 cycles of denaturation at 94ºC for 30 sec, annealing at 55ºC for 30 sec, and extension at 72ºC for 1.5 min, followed by final extension at 72ºC for 3 min (table 4.4.). The PCR products were analyzed by electrophoresis on 1.2% agarose gel containing 0.1 µg/ml ethidium bromide. DNA standard (100 bp DNA ladder) used to analyze the PCR products were purchased from New England Biolabs (Beverly, MA).

Table 4.4. PCR cycling protocol.

I	**Denaturation**		**94°C**	**1 min**
II	**33 cycles**			
		denaturation	**94°C**	**30 sec**
		annealing	**55°C**	**30 sec**
		extension	**72°C**	**1.5 min**
III	**Final extension**		**72°C**	**3 min**

4.2. EXPRESSION OF CA XII IN RAT TISSUES

4.2.1. RT-PCR

Smart RACE cDNA amplification kit (Clontech, Palo Alto, CA, USA) was used to clone rat CA XII. One microgram of kainic-acid treated rat brain mRNA was reverse transcribed with poly-d(T) primer and Powerscript reverse transcriptase. The reaction was done for 1.5 h at 42°C in a 20 µl volume, after which the obtained RT-PCR library was diluted with 50 µl of dilution buffer containing 10 mM Tricine-KOH and 0.1 mM EDTA. 5 µl of this library was used for each PCR reaction. Two primers for amplifying CA XII cDNA were designed based on the published mouse CA XII sequence in Genbank (accession numbers NM_178369: forward 5'-TGAGTCGCCAGGACAAAG-3' and reverse 5'-GATGTCTGAGACTGCCAG-3' (Sigma Genosys, Cambridgeshire, UK). The PCR reaction was denatured at 94°C for 30 s, annealed at 58°C for 30 s and elongated for 1 min at 72°C. The PCR reaction was amplified for 35 cycles. The fragments were analyzed in 1.2% agarose gel. The produced band was eluted from the gel with Qiaex II gel extraction kit (Qiagen, Chatsworth, CA, USA) and sequenced with the primers used for the PCR using BigDye terminator version 3.1 cycle sequencing kit. Sequencing analysis was performed with Abi 310 gene analyzer.

4.2.2. In situ hybridization and northern blotting

4.2.2.1. Animal treatments and tissues preparations

Adult male rats (Sprague-Dawley) were injected subcutanously with kainic acid (10 mg/kg; Sigma, St. Louis, MO or Ocean Produce Int., Canada) in 0.9% saline to provoke them a kainic acid induced status epilepticus. After kainic acid injection, first rats showed behaviour such as staring and movement arrest and thereafter wet dog shakes eventually leading to unilateral limb and/or facial clonus. On average after 1 h, rats had the first generalized tonic-clonic seizure with bilateral limb clonus (stage 3 seizure in Racine scale), which was considered as zero time point. Behavioural seizure activity slowly

changed continuously during several hours. The kainic acid injected animals were killed 1, 3, 6, 12, 24, 72 h or 7 d after the onset of the first typical tonic-clonic seizure.

For *in situ* hybridization histochemistry, the brains (n=4 for each time point) were removed and frozen on dry ice. Coronal sections of 14 µm were cut through the hippocampus with a cryostat and mounted onto Fisherbrand Superfrost Plus slides (Fisher Scientific, Pittsburgh, USA). Untreated animals (n=4) were used as controls. For Northern blot analysis, brains were cut with a cryostat between optic chiasma and anterior pons and were stored at - 80 ºC. Untreated animals were used as controls.

4.2.2.2. Extraction of mRNA

Adult rat (Sprague-Dawley) brains were homogenized in a solution of 200 mM NaCl, 200 mM Tris (pH 7.5), 1.5 mM $MgCl_2$, 2% SDS and 200 µg/ml proteinase K (Fermentas) in DEPC-treated water (diethylpyrocarbonate). The lysates in this solution were passed through a sterile 21-gauge needle four times and incubated at 45 ºC water bath for 1 h. NaCl was added to each sample to a final concentration of 500 mM and then the samples were passed through a 21-gauge needle four times. Thereafter oligo(d)T cellulose (75 mg; Invitrogen) was added to each sample and the samples were incubated for 1 h at room temperature. Then the samples were centrifuged at 8000 rpm for 10 min at 4 ºC and supernatant was removed. The pellets were washed two times in a buffer solution containing 500 mM NaCl and 10 mM Tris (pH 7.5) in DEPC-treated water and then three times in a buffer containing 250 mM NaCl and 10 mM Tris (pH 7.5) in DEPC-treated water. Thereafter the washed samples were replaced into spin-columns (Invitrogen) and washed four times with buffer containing 250 mM NaCl and 10 mM Tris (pH 7.5) in DEPC-treated water. The mRNA was eluted with 400 µl of buffer containing 10 mM Tris (pH 7.5) in DEPC-treated water. Then 60 IUs of RNAse inhibitor (Fermentas) and 5 IUs of RNAse free DNAse (Boehringer Mannheim) were added to each sample and then the samples were incubated for 30 min at 37 ºC. The mRNA was precipitated with ethanol, dissolved again in water and stored at -80 ºC until used.

4.2.2.3. Oligonucleotide probe preparation

Searches of the Genbank database revealed no significant homology of the oligonucleotide sequences with any other previously characterized transcript. The oligonucleotide probes were designed according to the sequences of carbonic anhydrases found in the Genbank. The oligonucleotides were labelled with ^{33}P-dATP (DuPont-NEN Research Products, Boston, USA) for *in situ* hybridization histochemistry and with ^{32}P-dATP for Northern blot hybridization using terminal deoxynucleotidyltransferase (Fermentas). The labelled oligonucleotides were purified with Qiaex II kit (Qiagen). The oligonucleotide probes used are listed in table 4.5.

Table 4.5. List of the oligonucleotides and primers used.

Gene	Sequence	Bases	Species
CA II	gggagcaaggatcaaagttagcaaaggctgcacgtttccc	516-555	rat
CA IV	gtccttggagtccgagtcctgcaccttatcgcctgttgtc	488-527	rat
CA VII	cgaaccatgtagagtgcatctgtcaggcggttcatgctcg	851-890	rat
CA VIII	ccctatctggacgaacagtgcaatgatgacaatgccgtgg	559-598	rat
CA XII	cttgtcactggcggtgctgaagtcagggtacaggtctgag	560-599	mouse
CA XII	tggtgatccttaagaagca	148-166	mouse
CA XII	gcacagggtttcggaaaact	821-840	mouse
CA XII	tgagtcgccaggacaaag	90-107	mouse
CA XII	gatgtctgagactgccag	1178-1195	mouse

4.2.2.4. In situ hybridization method

At least four sections for each subject were hybridized with each oligonucleotide probe. The sections were dried in air at room temperature and hybridized at 42°C for 12-18 h with a mixture of 4 X SSC, 50% formamide, 1 X Denhardt's solution, 1% sarcosyl, 0.02 M phosphate buffer (pH 7.0), 10% dextran sulphate, 500 µg/ml heat denaturated salmon sperm DNA, 200 mM dithiothreitol and 1 x 10^7 cpm/ml of the labelled probe. After hybridization, the sections were washed 4 x 15 min each in 1 X SSC at 55°C and thereafter left to cool for 1-3 h at room temperature. The sections were then dipped in distilled water and subsequently in 75 and 90% ethanol and air dried at room temperature. The sections were covered with Biomax MR (Kodak, Rochester, NY, USA)

and exposed for up to 6 months. It has been previously shown that hybridization with sense probes gives a negligible background (Zhang et al., 1998).

4.2.3. Northern Blotting

RNA samples (4 μg/lane) were separated on agarose gel containing formaldehyde, and were then transferred onto a nylon membrane (Hybond N+, Amersham) and crosslinked with UV-light stratalinker (Stratagene). The blotted membrane was prehybridized at 42°C for 60 min in a hybridization solution containing 4 X standard sodium citrate (SSC) (1XSSC,), 50% formamide, 5 X Denhardt's solution (1XDenhardt's,), 1% sodium dodecyl sulfate (SDS), 0.02 M phosphate buffer (pH 7.0), 10% dextran sulphate and 100 μg/ml heat denatured salmon sperm DNA. Thereafter the labelled probe was added to the hybridization solution to a final concentration of up to 5 x 10^6 cpm/ml, and the membrane was hybridized at 42°C overnight. The membrane was washed with increasing stringencies with maximal stringency of 2 X SSC at 65°C and was covered with Biomax MR (Kodak) autoradiographic film (Rochester, NY USA) and exposed for 3-7 days at -80°C. Cyclophilin oligonucleotide was used as an internal control to verify that equal amounts of mRNA were loaded into the gel. Northern blots CA VIII and CA XII were done sequentially using the same membrane. It was first hybridized with the CA XII probe. After exposure, the membrane was hybridized with the cyclophilin probe and exposed again. The CA XII signal was completely abolished by the cyclophilin hybridization and the subsequential washes. The membrane was then hybridized with the CA VIII probe and exposed. Therefore, the cyclophilin lane for CA VIII and CA XII is the same. Northern blots were quantified with UTHSCSA Image Tool 3.00 software (University of Texas Health Science Center, San Antonio). The integrated densities of each transcript were balanced against cyclophilin in the same lane and the values representing control and kainic acid treated animal were compared.

5. RESULTS

5.1. EXPRESSION OF *CA12* GENE IN MOUSE AND RAT TISSUES

The expression of *CA12* gene in mouse tissues was investigated by PCR amplification of a commercially available set of cDNAs produced for selected mouse tissues including the heart, spleen, lung, liver, skeletal muscle, kidney, testis, 7-day embryo, 11-day embryo, 15-day embryo and 17-day embryo, and in rat tissues by RT-PCR of the kainic-acid treated rat brain mRNA. Figure 5.1. shows a strong 700-bp band in kidney which served as a control in this investigation. A strong expression can be seen in the brain tissues. A weak expression could be noted in the testis and even fainter signal was seen in the lung. The heart, spleen, liver and skeletal muscle were negative. Embryos showed positive bands and the signal became stronger with increasing age of the embryo. This could indicate a role for CA XIII in developmental regulation (table 5.1.).

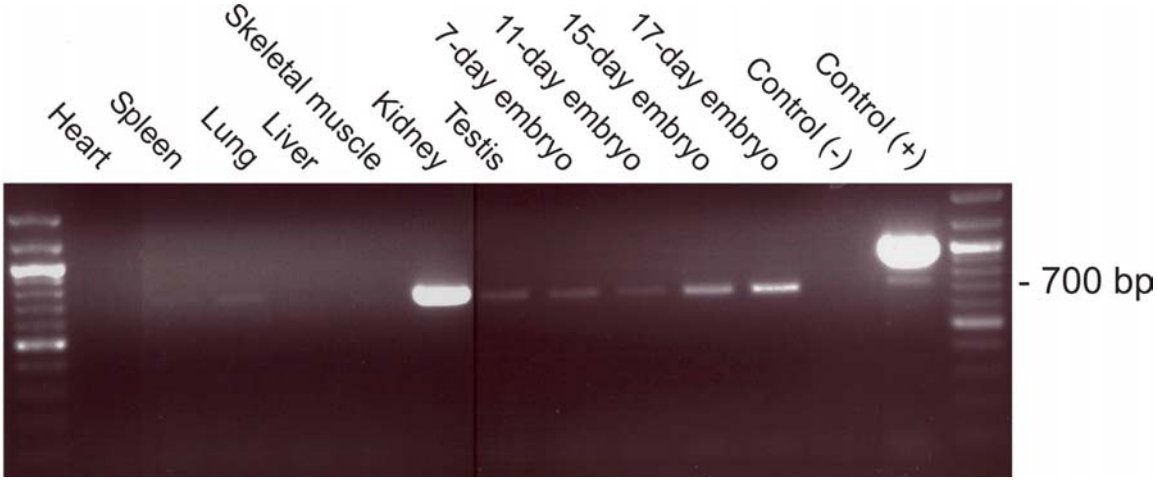

Figure 5.1. PCR-analysis of mouse CA XII mRNA expression. The strongest 700-bp signal is seen in the kidney, followed by the testis, embryos and lung. In embryos, the signal becomes more prominent at stages of 15 and 17 days.

The expression of the CA XII gene in the rat and mouse brain was investigated using RT-PCR amplification. The results showed positive bands of expected size in both species (data not shown). We further analyzed the product obtained from the rat brain cDNA by sequencing. The primer sequences were located just up- or downstream of the 5'- or 3'-untranslated region, respectively, so that they would amplify the whole open reading

frame. Because the amplified fragment was sequenced using the same primers, the utmost 5'- and 3'-ends were missed. The obtained 968 bp sequence (Accession # AY952140) was identical to one previously published cDNA sequence (Accession # XM_343416, Rattus norvegicus similar to 2310047E01Rik protein (LOC363085)) which encodes for an unspecified CA. The amplified fragment was also 94% similar to mouse CA XII (NM_178369).

Table 5.1. Expression of CA XII in mouse tissues by PCR analysis.

Tissue	Expression (+/-)
heart	-
brain	++
spleen	-
lung	+
liver	-
skeletal muscle	-
kidney	+++
testis	+
7-day embryo	++
11-day embryo	++
15-day embryo	++
17-day embryo	++

5.2. DISTRIBUTION OF CA XII PROTEIN IN MOUSE TISSUES

Immunohistochemical staining of CA XII revealed a distribution pattern that was limited to only a few mouse tissues. Kidney served as a positive control for the reagents, which labelled CA XII by immunofluorescence in the proximal tubules and in the intercalated cells of the collecting ducts (Kyllönen et al., 2003) (figure 5.2.). CA XII was located in the proximal convoluted tubule (PCT) and in the collecting ducts (CD) (A). Immunostaining was made also with normal rabbit serum (B) to show the negative control.

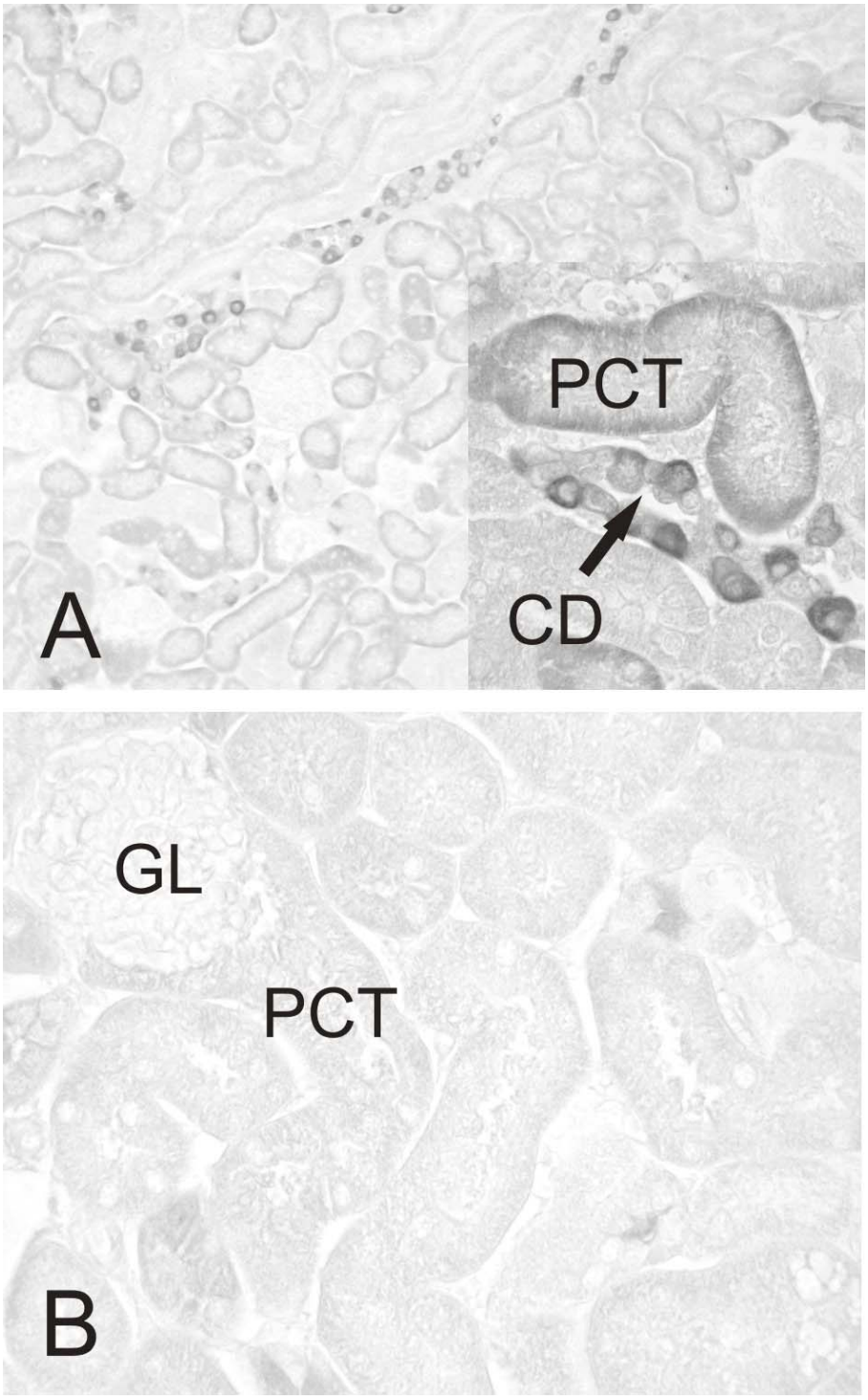

Figure 5.2. CA XII staining in mouse kidney in the proximal convoluted tubule (PCT) and collecting ducts (CD) (A). Immunostaining with normal rabbit serum served as a negative control (B). GL, glomerulus.

In the gastrointestinal tract, CA XII was not expressed in the stomach, duodenum and jejunum (figure 5.3.). The enterocytes in the ileum showed a faint positive staining and the reaction became much stronger in the colon and rectum. In the large intestine, the staining was most intense in the surface epithelial cuff region as described previously for human CA XII (Kivelä et al., 2000). The highest expression was seen on the basolateral surfaces of the epithelial cells.

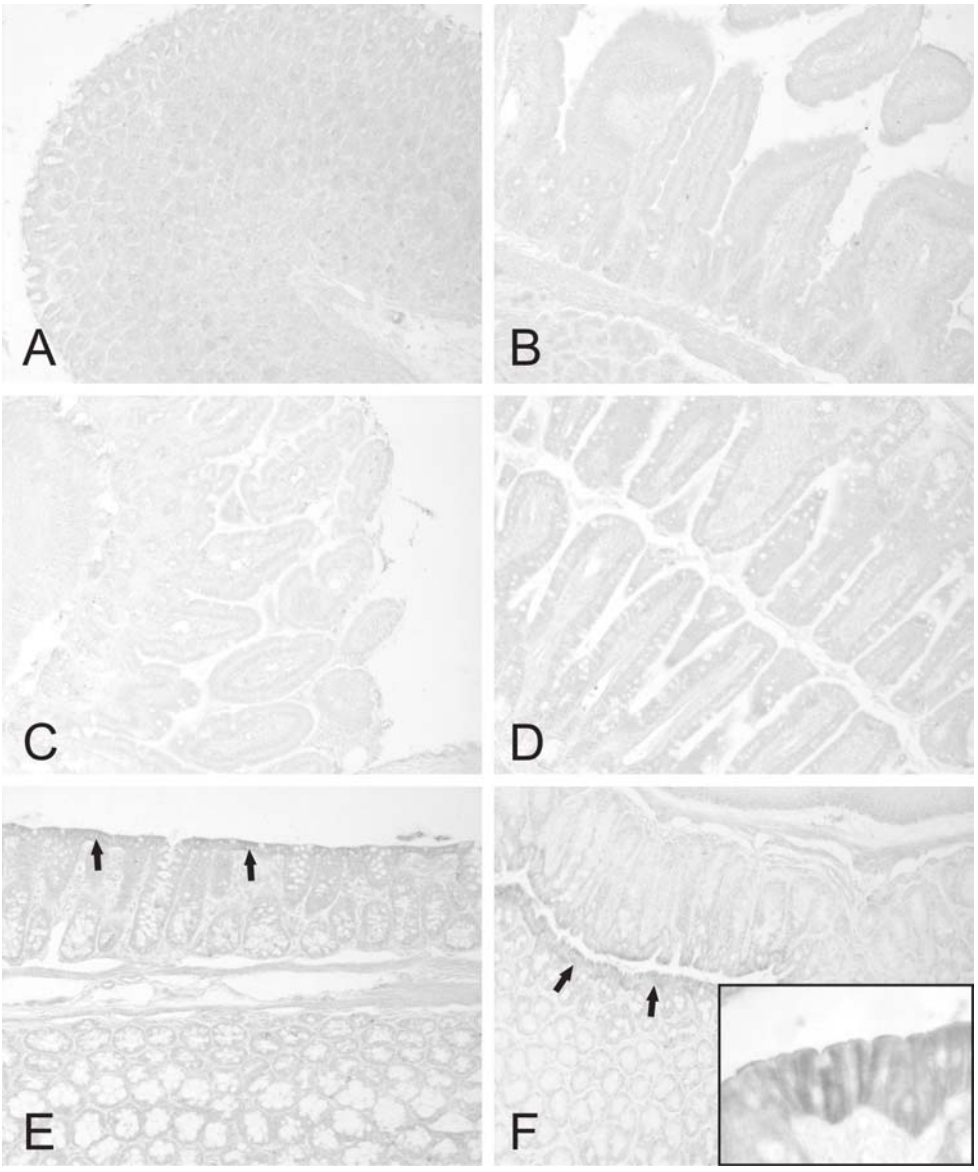

Figure 5.3. Immunostaining of CA XII in the mouse gastrointestinal canal. Negative staining can be seen in stomach (A), duodenum (B), and jejunum (C). Ileal enterocytes have stained faintly (D). A strong reaction is in the surface epithelial cuff region of the colon (E) and rectum (F). Insert of the panel F shows a basolateral surfaces of the enterocytes stained.

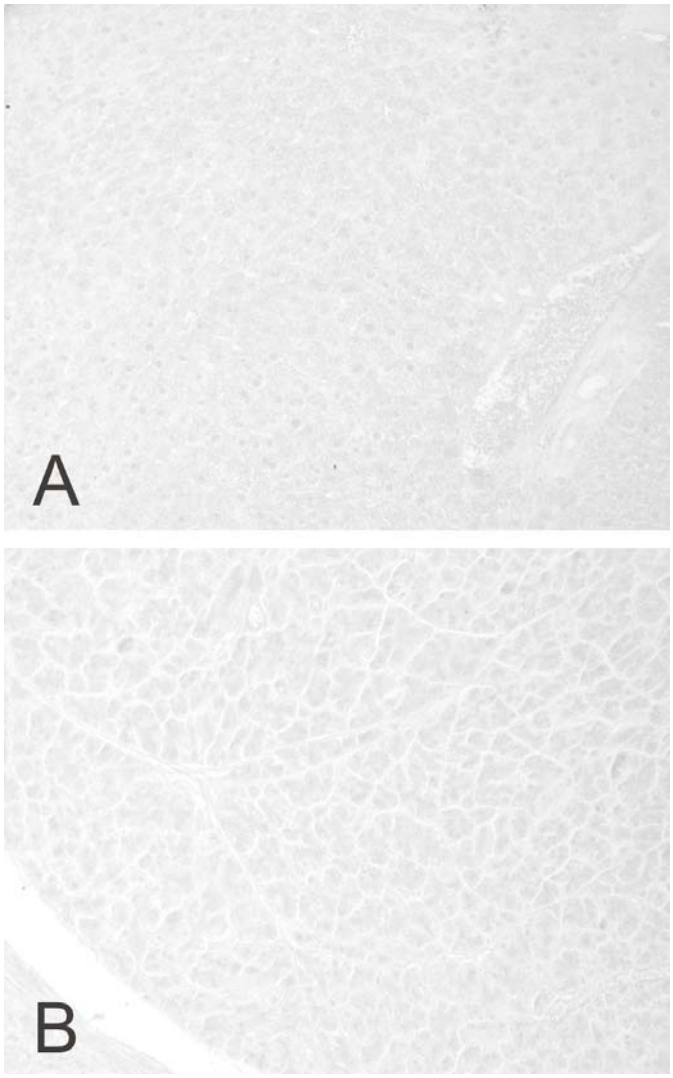

Figure 5.4. Immunostaining of CA XII in mouse liver (A) and pancreas (B). No reaction seen.

No specific staining for CA XII was found in the mouse liver and pancreas (figure 5.4.). Weak staining was detected in the developing sperm cells, in which the signal showed a crescentic or punctuate pattern, suggesting CA XII expression in the acrosomal membrane (figure 5.5.). No specific reaction was found in the psoas and heart muscle specimens (figure 5.6.).

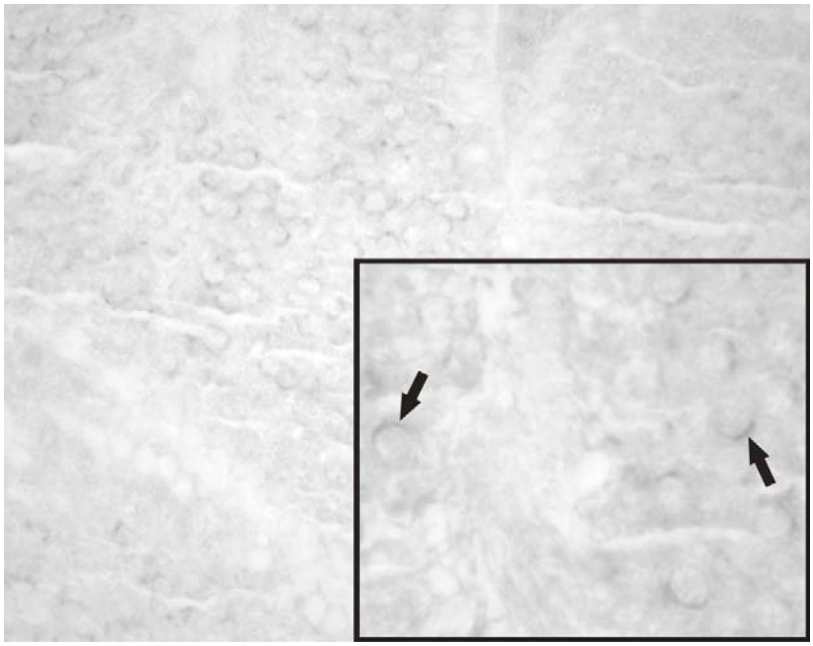

Figure 5.5. Immunostaining of CA XII in mouse testis. Weak staining can be seen in developing sperm cells probably at the site of the developing acrosome.

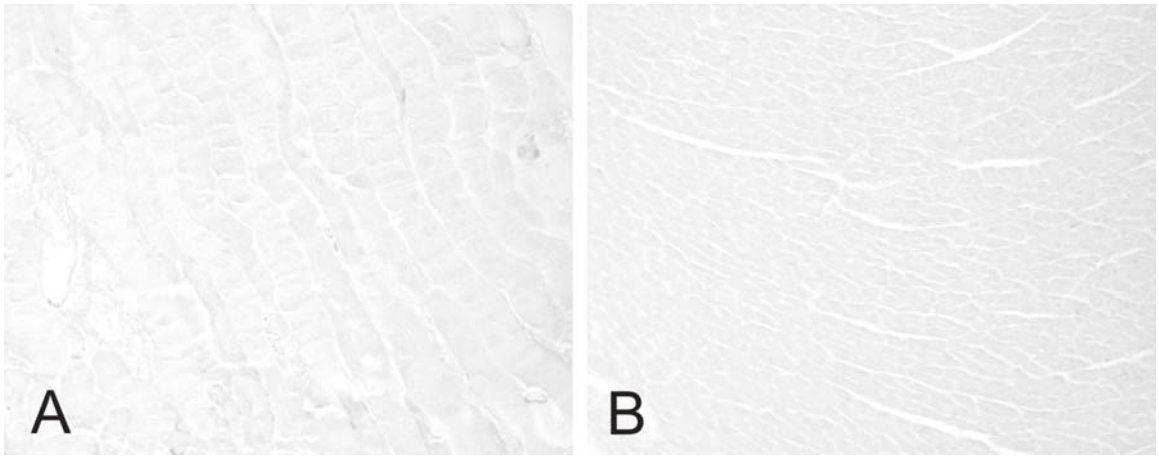

Figure 5.6. Immunostaining of CA XII in mouse psoas muscle and heart. No reaction seen.

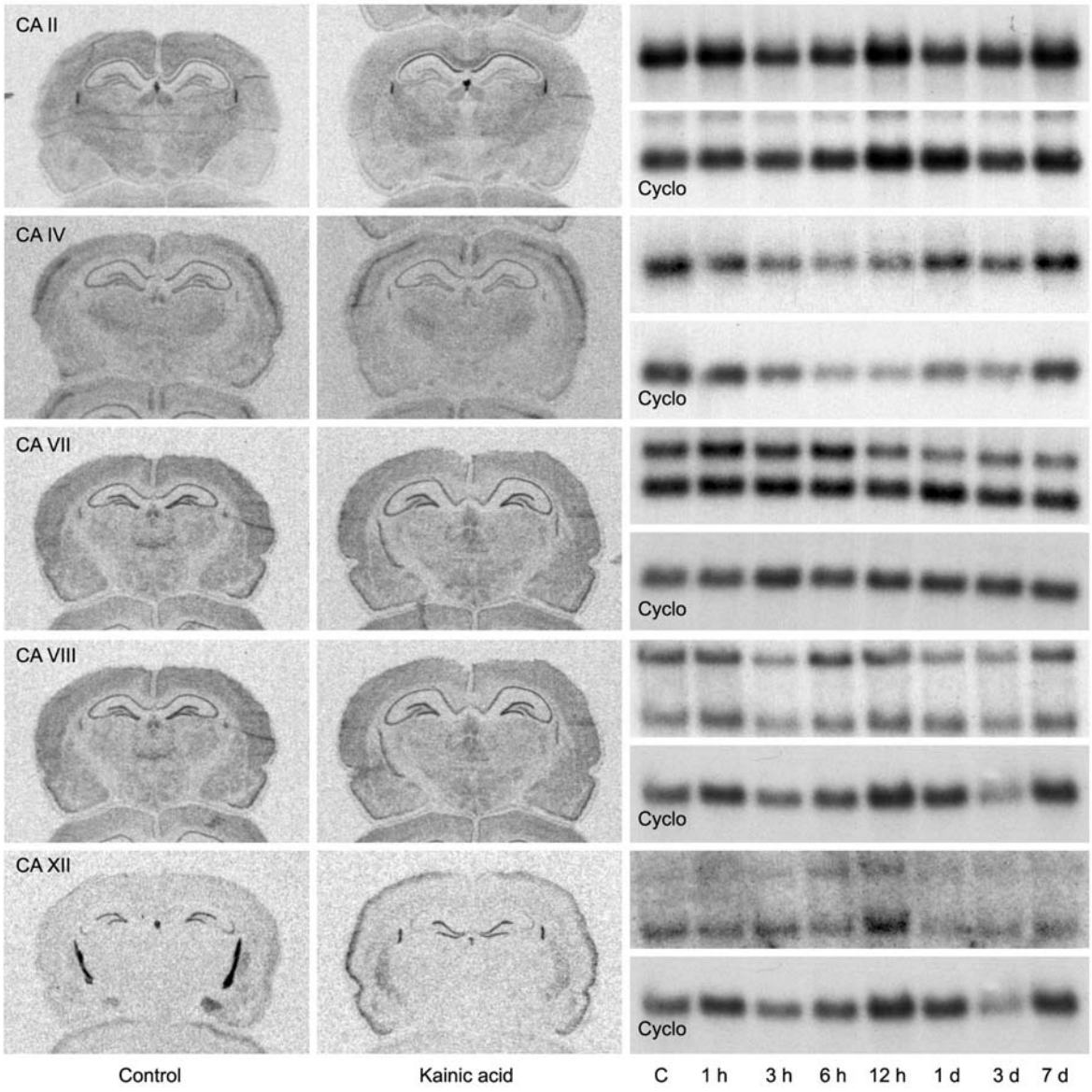

Figure 5.7. Expression of CA II, IV, VII, VIII and XII in rat brain in untreated animals and after kainic acid induced seizures. The brain section demonstrating the effect kainic acid on CA expression represents the 3 h (CA II) or 12 h (CA IV, VII, VIII and XII) time points.

5.3. *IN SITU* HYBRIDIZATION AND NORTHERN BLOTTING

To map the putative roles of the different CAs in CNS, we studied their expression in rat brain after kainic acid induced status epilepticus. As shown in figure 5.7., all the studied isozymes showed a clearly detectable basal expression in the brain. The expression of CA

II was diffusely localized in the cortex, striatum, hippocampus and midbrain structures. The expression of CA II was lower in the piriform and perirhinal cortex and in amygdala than in parietal cortical regions. CA IV expression was concentrated in layers III and VI in the cortex, hippocampus and thalamus. The expression of CA VII and CA-RP VIII was identical: relatively high level of expression was observed throughout the cortex, hippocampus and thalamus. The expression of CA XII differed significantly from the other CAs: relatively high levels of CA XII mRNA were seen in the dentate granule cells and medial amygdala. A detectable expression was also seen in the CA3 "tip" of the hippocampus. High level expression of CA XII – and also CA II – was seen in the choroid plexus.

After kainic acid induced seizures the mRNA levels of CAs IV, VII and VIII remained unchanged. The expression of CA II was selectively induced in the CA1 pyramidal cell layer of hippocampus 3-12 h after the onset of seizures. The CA XII mRNA levels were elevated similarly at 3-12 h throughout the cortical layer 1.

Northern blots of the CAs studied revealed expected sized bands: a 1,6 kb band for CA II, 1,6 kb band for CA IV, two bands of 1,0 and 2,2 kb for CA VII, 1,3 and 3,6 kb for CA-RP VIII, and 1,8 and 4,2 kb for CA XII. None of these clearly visible bands showed a demonstrative induction in any time points after the kainic acid treatment. CA XII appeared to be somewhat induced 12 h after administration of kainic acid, which is in line with the *in situ* data. The CA12 oligonucleotide was based on the mouse CA XII sequence. Comparison of the oligonucleotide sequence with the obtained rat CA XII sequence revealed four mismatches. This probably reflected to the Northern blot intensity, which was much lower for CA XII than for the other oligonucleotides.

6. DISCUSSION

Carbonic anhydrase XII is one of the most interesting α-CA gene family members. It is an active transmembrane protein with so far known limited distribution both in human and mouse tissues. CA XII has been related to several malignant tissues and it might work as a biomarker for some malignant tumors (non-small cell lung carcinoma) and it could be considered also as a potential target for novel therapeutic applications (Ivanov et al., 1998). The investigation of CA XII has been concentrated much on human tissues, and only this study has revealed some new facts of its distribution and expression in rodents.

6.1. EXPRESSION OF CA XII IN MOUSE TISSUES

The pattern of limited CA XII expression was shown in both immunohistochemical and PCR analyses. The highest amount of CA XII seems to be present in the kidney which was used as a positive control tissue based on the studies by Kyllönen et al. (2003). This is in line with the findings by Türeci et al. (1998) and Ivanov et al. (1998), who showed high CA XII mRNA expression in the human kidney and renal tumors. Based on the studies by Ivanov et al. (2001), the strongest signal for human CA XII mRNA was reported in the kidney and the second strongest in the colon.

The immunohistochemical staining revealed that CA XII protein is located at the basolateral plasma membrane of certain epithelial cells in both the human colon and kidney (Kivelä et al., 2000; Parkkila et al., 2000). CA XII has a similar pattern of expression in the mouse colon to that previously reported in the human colon (Kivelä et al., 2000). The localization of CA XII in the mouse kidney was clearly different from that reported previously for the human kidney. In the latter, CA XII is localized to the proximal and distal tubules and principal cells of the collecting ducts (Parkkila et al., 2000), whereas in the mouse, it was found in the proximal tubules and intercalated cells of the collecting ducts (Kyllönen et al., 2003). The physiological roles of these differences are not yet clear. On the other hand, the conservation of CA XII expression in

the colonic enterocytes in both species suggests that the enzyme serves a similar role in the human and mouse gut.

It is well known that the colonic enterocytes are equipped with a number of ion channels, carriers, and pumps, allowing highly efficient transport of salt and water. The enterocytes located in the epithelial cuff region are differentiated from the colonic crypt cells (Lipkin, 1985). The base crypt enterocytes show the highest proliferative activity, demonstrate limited expression of differentiation markers, and have a high chloride secretory activity (Kunzelmann and Mall, 2002). The most luminal epithelial cells, representing the mature enterocytes, have a lower tendency to proliferate, express several differentiation marker proteins and certain lectins, and have a primarily absorptive function. The colonic absorption of NaCl can be electrogenic via epithelial Na^+ channels or is electroneutral via parallel Na^+/H^+ and Cl^-/HCO_3^- exchange (Kunzelmann and Mall, 2002). From these two pathways, electroneutral NaCl absorption has been shown to take place in both crypts and surface epithelial cuff region. In contrast, electrogenic absorption via Na^+ channels is mainly located at the epithelial cuff region that is also the site of CA XII expression. Unfortunately, the role of CAs in electrogenic NaCl absorption is largely unknown. On the other hand, the participation of the colonic CA in the electroneutral NaCl absorption has been well established by CA inhibitors (Charney et al., 1986). Here, CA has been shown to be involved in the absorption of NaCl via the synchronous operation of apical Na^+/H^+ and Cl^-/HCO_3^- exchange processes. CAs also participate in the alkalization of the luminal contents by generating HCO_3^- for apical Cl^-/HCO_3^- exchange (Feldman, 1994).

Interestingly, recent results have indicated that CAs can be physically and functionally associated with Cl^-/HCO_3^- (AE1) and Na^+/H^+ exchangers (Sterling et al., 2001; 2002; Li et al., 2002). So far, CA IV is the only membrane-bound CA isozyme which has been shown to interact with AE1 protein (Sterling et al., 2002). It is quite possible that CA XII also could drive ion exchange processes across the colonic plasma membranes via a direct link with an ion transport protein.

Two major techniques have been used to define the physiological role of CA isozymes; a membrane-permeable or –impermeable CA inhibitor and knock-out mouse models deficient in one CA isozyme. The inhibitor studies often hamper by lack of isozyme-specificity. Two knock-out mouse colonies with CA deficiency have been reported: CA II-deficient mice produced by chemical mutagenesis (Lewis et al., 1988) and CA IX-deficient mice developed by targeted mutagenesis and homologous recombination (Ortova Gut et al., 2002). The physiological studies of renal and colonic function in CA XII-deficient mice would be quite interesting because one could predict that disruption of CA XII function would result in abnormal regulation of ion and pH homeostasis, because both kidney and colon play key roles in the maintenance of body electrolyte and pH balance.

Both of these organs also express other CA isozymes such as CA II, CA IV and CA XIV (Zhu and Sly, 1990; Parkkila et al., 1994; Fleming et al., 1995; Parkkila et al., 2002, Kyllönen et al., 2003). These isozymes can compensate for the loss of one isozyme following a gene disruption. Although this potential functional redundancy makes predicting the phenotype of the mouse with a targeted gene disruption less straightforward, establishing whether and which CA genes are upregulated would provide a clue as to the functional importance of the disrupted gene.

6.2. EXPRESSION OF CA XII IN RAT BRAIN

Carbonic anhydrases are believed to play a central role in several functions of CNS. For example, CA II is involved in CSF secretion and glial function, CA IV is a protein component in the blood brain barrier, CA VII may modulate GABAergic responses, and CA XIV has been proposed to participate in excitatory synaptic transmission.

In the present studies were found a high basal expression for each isozyme in the rat brain. CA IV, CA VII and CARP-VIII were expressed throughout the cortex, thalamus and hippocampus. The labelling pattern in these structures resembled that of genes

expressed preferentially in neurons. Since kainic acid did not cause any change in the level of their expression, it can be said that their expression is very stable. In contrast, CA II and CA XII expression were induced by kainate. The expression of CA II was somewhat diffuse in the cortex and midbrain but different from that of CA IV, CA VII and CARP-VIII. These genes were not expressed in the striatum, whereas CA II showed a relatively high expression in the white matter. This type of basal expression was expected, since CA II is preferentially expressed in oligodendrocytes (Cammer & Zhang, 1991; Ghandour et al., 1980; Ghandour & Skoff, 1991; Kumpulainen et al., 1983; Kumpulainen & Korhonen, 1982; Roussel et al., 1979), in astrocytes (Cammer & Tansey, 1988; Cammer & Zhang, 1991; Kimelberg et al., 1982; Roussel et al., 1979) and in neonatal rats in microglia (Cammer & Zhang, 1996).

CA II participates in the production of CSF by regulating transport of bicarbonate, sodium and water from circulation to the CSF (Brown et al., 2004), so the expression of CA II in the choroid plexus is expected. Very unexpected was the fast induction of CA II in the hippocampus. Oligodendrocytes express kainate/AMPA receptors (Patneau et al., 1994), but by 3-12 h there is virtually no reactive glia in the CA1 region. The uniform labelling of the CA1 pyramidal cell layer suggests mainly neuronal expression. Kainic acid induces selective neuronal death in the CA1 region, but also sprouting in the surviving neurons (Perez et al., 1996; Smith & Dudek, 2001). At 3-12 h, when CA II was induced in the CA1 cells, the neurons are still viable, but starting to commit the pathway eventually leading to programmed cell death. At this time, pro-apoptotic as well as neuroprotective genes are induced. However, the function of CA II has not been associated with sprouting, apoptosis or cellular stress. Interestingly, CA II deficient mice are more resistant to seizures. Mortality to experimental seizures is also decreased (Velisek et al., 1993). The putative role of CA II in the CA1 pyramidal cells might therefore be pro-epileptic and possibly neurodegerative. This hypothesis is supported by the fact that two used antiepileptic drugs, topiramate and valproic acid, inhibit CA II (Casini et al., 2003; Masereel et al., 2002).

This study shows the first evidence that mRNA for membrane bound CA isozyme, CA XII, is also expressed in the rodent brain. CA XII mRNA is expressed in choroid plexus, striatum, CA3 and dentate granule cell layer of the hippocampus. Kainic acid treatment induced a selective and rapid expression of CA XII mRNA in the cortical layer I. The specific signal induced CA XII in this layer only and the consequences of the observed induction are not known because usually kainic acid administration produces only few changes in this particular cortical layer. There is no cell death in this area, and induction of immediate early genes (IEGs) or stress genes such as those encoding heat shock proteins are not exhibited. There is no exact physiological significance to this phenomenon.

Topiramate, a commonly used antiepileptic drug, is a very potent, nanomolar inhibitor of CA XII (Vullo et al., 2005a). As CA XII mRNA expression in the brain cortex was markedly stimulated by the kainic acid treatment, it could be an interesting hypothesis that CA XII expression might be functionally linked to epileptogenesis. CA XII could be a protein target of topiramate, and inhibition of CA XII enzymatic activity might be beneficial for the desired outcome of antiepileptic therapy.

CAs have probably an important role in the regulation of CSF secretion and ion concentration and the main evidence for this is that acetazolamide can reduce CSF production by 30-50 per cent (Swenson, 2003). By histochemical staining methods CA activity have been demonstrated in the cytoplasm and plasma membranes of the rat choroid plexus cells (Masuzawa et al., 1981). Immunocytochemical staining results have provided further evidence that these cells express at least two cytosolic isozymes, CA II and CA III (Kumpulainen & Korhonen et al., 1982; Norgadi et al., 1993). CA II as a high activity isozyme could be the main catalyst in the intracellular reaction in which bicarbonate and protons are formed from carbon dioxide and water.

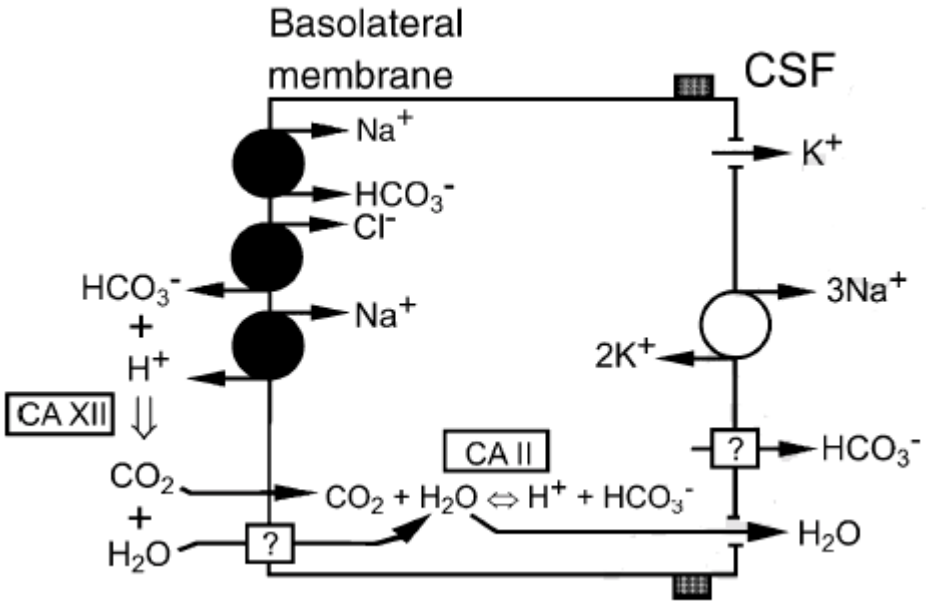

Figure 6.1. A hypothetical model of ion, carbon dioxide and water transport mechanisms in the rat choroid plexus. Based on the results CA II and CA XII are probably the main catalysts in the chemical reactions which facilitate secretion of bicarbonate and water into the CSF. (Modified from Swenson, 2003 and Brown et al., 2004)

CA XIV was the first membrane-bound isozyme which was demonstrated in the choroid plexus with a very restricted expression (Parkkila et al., 2001). The present results showed that CA XII mRNA is highly expressed in the rat choroid plexus. Since CA XII is confined to the basolateral plasma membrane in other tissues such as kidney and colon (Parkkila et al., 2000; Kivelä et al., 2000), the protein could be expressed on the basolateral surfaces also in the choroid plexus epithelium. Because the active site of CA XII is located on the cell exterior, this isozyme might contribute to recycling of carbon dioxide into the epithelial cells facilitating transport of water and bicarbonate into the CSF. Due to recent studies acetazolamide seems to be a strong inhibitor of CA XII (Vullo et al., 2005a). Based on our results it is conceivable that the beneficial effect of acetazolamide in patients with elevated intracranial pressure is attributable to inhibition of both CA II and CA XII locating in the chroid plexus epithelium.

7. CONCLUSIONS

The expression of carbonic anhydrase isozyme XII in mouse tissues was the first goal of this work and it revealed a very limited distribution pattern. The highest expression was seen in the kidney which served as a positive control. The enterocytes of the ileum showed faint staining which became stronger in the colon and rectum, and in the large intestine the staining was most intense in the surface epithelial cuff region as predicted. Rest of the gastrointestinal tract (stomach, duodenum, and jejunum), liver, pancreas, psoas muscle and heart showed no specific reaction. The developing sperm cells showed weak crescentic staining in the acrosomal membrane.

The *CA12* gene expression was investigated by PCR and it confirmed the strong expression in the kidney. Lung tissues also showed some expression, but heart, spleen, liver, and skeletal muscle showed no reaction. However, embryos (7-, 11-, 15, and 17-day) showed positive expression and it became stronger with increasing age of the embryo. This is very interesting observation, and might be worth of a new investigation.

Secondly, CA XII was examined in rat brain tissues after kainic acid induced status epilepticus by *in situ* hybridization. In the brain CA XII mRNA seems to have a detectable basal expression. The expression pattern of CA XII is very different from the other CAs used in the experiment. High levels of CA XII were observed in the dentate granule cells, medial amygdale and choroid plexus, and detectable expression was also seen in the CA3 “tip” of the hippocampus. After the kainic acid induced status epilepticus CA XII mRNA levels elevated at 3-12 h throughout the cortical layer. Northern blot analysis gave a 1,8 and 4,2 kb bands for CA XII but it showed no clear induction in any time points after the kainic acid treatment. CA XII appeared to be somewhat induced 12 h after administration of kainic acid, which is in line with the *in situ* data. The CA12 oligonucleotide was based on the mouse CA XII sequence. Comparison of the oligonucleotide sequence with the obtained rat CA XII sequence revealed four mismatches. This probably reflected to the Northern blot intensity, which was much lower for CA XII than for the other oligonucleotides.

Hypothetically, CA XII could be expressed on the basolateral surface of the rat choroid plexus epithelium, located on the cell exterior. There CA XII migth participate in recycling of CO_2 ($HCO_3^- + H^+ \rightarrow CO_2 + H_2O$) facilitating the transport of water and bicarbonate molecules across the basolateral surface.

Based on studies with rat brains, CA XII expression might be functionally linked to epileptogenesis and it could be a protein target of an antiepileptic drug, topiramate. Inhibition of CA XII enzymatic activity could be beneficial for the desired outcome of antiepileptic therapy. Recent studies show that acetazolamide seems to be a strong inhibitor of CA XII. Our results demonstrate that it is conceivable that the beneficial effect of acetazolamide in patients with elevated intracranial pressure is attributable to inhibition of both CA II and CA XII locating in the chroid plexus epithelium.

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