Chapter 2
Physiological Functions of the Alpha Class of Carbonic Anhydrases

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Abstract Carbonic anhydrases are ubiquitous enzymes that catalyze the reversible hydration of carbon dioxide. These enzymes are of ancient origin as they are found in the deepest of branches of the evolutionary tree. Of the five different classes of carbonic anhydrases, the alpha class has perhaps received the most attention because of its role in human pathology. This review focuses on the physiological function of this class of carbonic anhydrases organized by their cellular location.

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1 Introduction

Carbonic anhydrases catalyze the reversible hydration of CO_2 ($CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$), which allows this enzyme to regulate intra- and extra-cellular concentrations of CO_2, H^+, and HCO_3^-. Decades of research have implicated CA in a broad range of physiological processes including gas exchange at the air water interface, transport of CO_2 and HCO_3^- across membranes, biosynthetic reactions in metabolically active tissue, acid–base balance, secretion, calcification, signal transduction, oncogenesis, proliferation, among the many that have been reported [1–16]. These seemingly disconnected functions are mediated by specific isoforms in the α-CA family. Sixteen members of this family have been identified which have distinct tissue-specific expression, kinetic properties, and sensitivity to inhibitors [17]. It appears unlikely that this family will be expanded further as searches of genomic databases have not identified any additional CA sequences [18]. Among those identified, there are eight cytosolic proteins (CA I, CA II, CA III, CA VII, CA VIII, CA X, CA XI, CA XIII), two mitochondrial matrix proteins (CA VA, CA VB), one secreted protein (CA VI), two glycosylphosphatidylinositol (GPI)-anchored proteins (CA IV and CA XV), and three transmembrane proteins (CA IX, CA XII, CA XIV). Three of the cytosolic isoforms (VIII, X, and XI) have no activity as they lack one or more of the histidine residues that coordinate the zinc ion in the catalytic pocket. As a group, these are called CA-related proteins and appear to be expressed exclusively in the brain [19]. The other isoforms have varied activities based on the efficiency of proton transfer, differences in active site residues, quaternary structure, and potentially localization [17, 20–22]. In this chapter, the physiological role of the catalytically active forms of CA will be discussed from the perspective of location: cytosolic, mitochondrial, secretory, and membrane-associated.

2 Cytosolic CAs

The role of carbonic anhydrase in CO_2 excretion is well known. In red blood cells (RBCs), CA activity accelerates the rate of conversion between molecular CO_2, which easily diffuses across membranes, and HCO_3^-, the form in which the majority of CO_2 is transported in the circulation. CO_2 produced by tissues diffuse into RBCs where it is hydrated to form bicarbonate ions that are transported via the band 3 anion exchanger and protons that are buffered by hemoglobin. The reverse occurs at gas exchange organs where HCO_3^- is dehydrated producing CO_2 that then diffuses across the water/air interface down its partial pressure gradient. RBC CA is indirectly related to O_2 loading and unloading through the Bohr effect [23] [reviewed in [24, 25]]. Mammalian RBCs express both CA II and CA I [25, 26]. It is thought that CA II activity dominates because of its fast kinetics, although the intracellular microenvironment may influence how these enzymes operate in vivo. On the other hand, it is widely accepted that CO_2 excretion in vertebrates is not limited by RBC CA activity [1]. Further details on this topic can be found in Chap. 18 (Swenson).

Hemolytic anemia is a disease in which RBCs are destroyed prematurely which leads to anemia. Glucose-6 phosphate dehydrogenase deficiency induces hemolytic anemia [27]. These patients have significantly lower CA I expression compared to control patients [10]. It is postulated that this is related to the rate of synthesis of CA I relative to hemoglobin since data are normalized to hemoglobin content. That said, CA II expression is increased, as is total CA activity. While CA I has substantially lower activity than CA II, which is the more physiologically relevant isoform, CA I expression may serve as a marker for hemolytic anemia.

CA II is also highly expressed in kidney intercalated cells and at lower levels in the proximal tubules, loop of Henle, and collecting duct principal cells [28, 29] where CA II regulates bicarbonate flux. CA II deficiency is an autosomal recessive trait characterized by renal tubular acidosis, osteopetrosis, cerebral calcification, and growth retardation [30]. A mouse model has been developed which partially mimics the human disease [31]. Kidneys of these mice are virtually devoid of medullary collecting duct intercalated cells [32] where CA II expression is normally high. Interestingly, these cells are present at birth, but at some point during post-natal development, intercalated cells are selectively removed in the medullary collect ducts and replaced by principal cells. This suggests that CA II may play a role in regulating cell-type diversity in kidney collecting ducts. Indeed, chronic acetazolamide treatment of adult rats causes significant remodeling of the cellular profile of collecting ducts [33]. This may represent an adaptive process to correct or stabilize the metabolic acidosis that would otherwise ensue following loss of CA II function.

In addition to its ability to mediate the reversible hydration of CO_2, CA II appears to interact with a variety of membrane-bound carriers to balance cytoplasmic pH. Examples of these include the chloride/bicarbonate exchanger AEI [34, 35], the sodium bicarbonate cotransporter NBC1 [36, 37], and the sodium/hydrogen exchanger NHE1 [38]. These interactions increase the activity of the transporters and have been coined “transport metabolons” [35]. Specific amino acid motifs, along with individual residues, have been identified that are required for the binding of the metabolon partners [39, 40]. Post-translational modifications have also been implicated in these interactions. For example, phosphorylation of NHE1 in the C-terminal cytoplasmic tail significantly increases the interaction with CA II and thus its activity [39]. Metabolons may also play a role in human pathologies. For instance, the interactions between CA II and NHE1 and AE3 have been implicated in cardiomyocyte hypertrophy [7]. In addition to the above transporters, CA II also interacts with members of the monocarboxylate transporter family (MCT1 and MCT4) and increases their activity, leading to enhanced export of lactate from Xenopus oocytes [41, 42] and astrocytes [43]. Protons, provided by CA II, are cotransported by the MCTs leading to the hypothesis that CA II acts as a “proton collecting antenna” [44]. In contrast to other transport metabolons, the interaction between CAII and the MCTs does not require the catalytic activity of CA II but rather its ability to shuttle protons via the proton wire, with residue His64 playing a central role [44, 45]. These features are described in more detail in Chap. 7 (Becker et al.).

CA III has several characteristics that distinguish it from other isozymes. Expression of CA III is remarkably high in skeletal muscle [46] and adipose, both

white [47] and brown [48]. However, CA III activity is low, at only 3 % of that of CA II [17]. This difference in activity has led to the idea that CA III may play a different role in cellular function beyond is catalytic activity. CA III has two reactive sulfhydryl groups that can reversibly bind to glutathione through disulfide bonds [49, 50]. This reaction would likely protect cells from irreversible protein oxidation [51]. Indeed, overexpression of CA III in cells protects them from H_2O_2-induced apoptosis [52]. Further, aged rats showed increased tissue levels of irreversibly oxidized CA III associated with decreased glutathione concentrations [53]. These data suggest that CA III might protect cells from oxidative damage [54]. However, muscle tissue in the CA III global knockout mouse responded no differently than muscle in wild type mice in response to hyperoxic challenge or muscle fatigability [55]. In fact, these authors showed that CA III expression is not required for normal growth, development, or life span of the mouse.

Adipose tissue stores fat and is central to energy homeostasis [56]. New adipocytes arise from precursors called adipose-derived stems cells or pre-adipocytes in a process called adipogenesis. Light and electron microscopy have revealed that these cells arise from perivascular sites [57–59]. Several studies have now shown that perivascular cells isolated from adipose have the ability to differentiate [60–65]. Importantly, the nuclear hormone receptor peroxisome proliferator-activated receptor γ2 (PPARγ2) that acts as the master regulator of adipogenesis is found in these precursor cells [66]. CA III expression is induced during adipogenesis [67] and possibly provides HCO_3^- to acetyl CoA carboxylase [68], the rate determining step in fatty acid biosynthesis. However, CA III is down-regulated in obese states [69] in the face of enhanced fatty acid biosynthesis [70]. This questions a role in substrate metabolism. Yet, recent data reveals that CA III regulates adipogenesis at the level of PPARγ2 gene expression [71]. While no changes in adipose content were noted in the CA III knockout mouse [see above [55]], Mitterberger et al. have shown that adipogenesis is enhanced in mouse embryonic fibroblasts (MEFs) isolated from CA III knockout mice [71]. This was associated with a 1000-fold increase in PPARγ2 expression. This suggests that CA III expression exerts a negative effect on PPARγ2 expression. Despite the fact the CA III expression is increased during adipogenesis as mentioned [67], it apparently is not required for the normal terminal differentiation of adipose tissue. Rather, it appears that CA III controls steps early in the differentiation process. Still unknown is the mechanism by which CA III regulates PPARγ2 expression and whether it serves a similar role in muscle during development. As noted above, CA III may play a protective role in oxidative damage. PPARγ2 also plays a role in oxidative stress. It has been shown that pharmacological activation of PPARγ2 attenuates the production of reactive oxygen species (ROS) in 3 T3-L1 adipocytes and in the insulin-resistant leptin deficient ob/ob mouse [72]. Thus, CA III may provide long term regulation during adipogenesis and protection in response to oxidative stress.

Carbonic anhydrase VII is one of the least characterized of the CA family. The human form was identified through genomic screening [73]. While it was predicted to have CO_2-hydrase activity, this was proven later using mouse [74] and human [75] recombinant proteins. The human form has catalytic activity that is close to

that of CA II [75]. It also has the highest esterase activity among the CA family members [75]. There are two forms of this protein: the long form is the predominant form and the shorter form is missing 56 residues at the N-terminus [76]. Based on western blotting, the protein is primarily expressed in colon, liver, and skeletal muscle, although it is also noted in brain [76]. Similar to CA III, it has two reactive cysteines and can be glucothionylated [75] suggesting a role as an oxygen free radical scavenger. CA VII has also been implicated in neuronal excitation by providing HCO_3^- which can mediate current through channels coupled to $GABA_A$ receptors [77]. This activity is suppressed when treated with membrane-permeant sulfonamides, supporting the hypothesis that CA VII plays a role in neuronal excitation and seizures [78]. Kaila and Ruusuvuori discuss this in further detail in Chapter 14. In addition, CA VII may play a role in neuropathic pain as acetazolamide in combination with midazolam treatment synergistically reduces neuropathic allodynia after spinal nerve damage [79]. In that regard, CA VII may represent a new drug target for managing neuropathic pain.

Human CA XIII isozyme was identified and characterized in 2004 [80]. In this study, the authors showed that CO_2 hydration activity is similar to that of CA I and CA V, each of which are characterized as having moderate catalytic activity. Inhibition profiles are similar to CA II [81]. CA XIII was localized to several tissues including the thymus, kidney, submandibular gland, small intestine, and notably in reproductive organs of both sexes [80]. Since pH and ion balance are likely to be tightly regulated in reproductive organs to ensure normal fertilization [80], it is surmised that CA XIII may contribute to reproductive processes by controlling optimal HCO_3^- concentration and pH homeostasis for the maintenance of sperm mobility. One could also postulate that CA XIII might contribute to normal fertilization process by producing the appropriate bicarbonate concentration to alkalinize the cervical and endometrial mucus [82]. CA XIII deficient animals are not yet available so testing these hypotheses must wait. However, there are data regarding a role of CA IV in bicarbonate-mediated activation of mouse and human sperm [83], an enzyme that will be discussed later in this chapter and Chap. 9 (Sly and Waheed). With renewed interest in tumor-associated CAs, Kummola et al. have demonstrated that CA XIII, along with two other cytosolic CAs (CA I and CA II), is down-regulated in colorectal cancer [82]. Because these three CAs genes are closely linked on chromosome 8, these authors suggest that down-regulation is related to reduced levels of a common transcription factor. The physiological reasons for down-regulation are left to speculation at this point.

3 Mitochondrial CAs

Chappell and Crofts demonstrated that HCO_3^- was impermeant to the inner mitochondria membrane [84]. While Elder initially proposed that HCO_3^- could provide the counter ion for energy-dependent Ca^{2+} transport [85], shortly thereafter it was shown that CO_2, not HCO_3^-, served this function [86]. With the advent

of molecular technology, we now know that the bicarbonate transporter family (SLC4A) includes 11 members (see http://slc.bioparadigms.org), none of which are located in the inner mitochondrial membrane. Thus, de novo synthesis of HCO_3^- within the mitochondrial compartment is required for providing substrate for pyruvate carboxylase in the gluconeogenic pathway and carbamoyl phosphate synthetase I in ureagenesis in the liver [87, 88].

The first mitochondrial CA was isolated from guinea pig liver and called CA V [89]. It was subsequently identified in mouse, rat, and human through molecular cloning [90–92]. That the transcript for mouse CA V was only identified in liver [90] while a wider distribution was suggested by western blotting [91], led to a search of the EST database revealing that there were two mouse mitochondrial CA sequences. Ultimately, these sequences were named CA VA and CA VB, respectively, and northern and western blotting revealed a significantly different tissue-specific distribution pattern between the two [93]. Interestingly, the human ortholog for the *CA5B*, which also has broad tissue expression, has been mapped to chromosome Xp22.1 [94] while *CA5A* was mapped to chromosome 16q24 [95].

Carbamoyl phosphate synthetase I utilizes HCO_3^- rather than CO_2 for the synthesis of carbamoyl phosphate [96]. This is the committed step in ureagenesis. Ornithine transcarbamylase utilizes carbamoyl phosphate as a co-substrate in the synthesis of citrulline [97–99], which is the first intermediate of the urea cycle. Dodgson et al. demonstrated that the synthesis of citrulline could be blocked by acetazolamide in guinea pig liver mitochondria [100]. Indeed, the inhibition curve for citrulline synthesis was identical to the inhibition curve for mitochondrial CA (CA VA). This was the first physiological evidence that carbonic anhydrase enhances access of HCO_3^- to the synthetase reaction, so CA must be considered a participant in ureagenesis. These studies raised the possibility that HCO_3^- created in the CA V reaction could drive other biosynthetic reactions, particularly that of the carboxylase family of enzymes. Pyruvate carboxylase mediates the first reaction in gluconeogenesis from pyruvate. Dodgson and Forester showed that pyruvate carboxylase activity was blocked by ethoxzolamide, a membrane permeant sulfonamide, in mitochondria isolated from liver from starved guinea pigs [87]. While earlier studies had suggested that sulfonamides inhibit pyruvate carboxylase directly [101], Dodgson and Forester showed that the inhibitory effect of ethoxzolamide on pyruvate carboxylase activity was lost in experiments where guinea pig liver mitochondria were pretreated with digitonin, in the presence of high bicarbonate, to compromise membrane integrity. These and other data suggest that the effect of ethoxzolamide is on mitochondrial CA, not pyruvate carboxylase. Dodgson and Forester also demonstrated that glucose production in hepatocytes was blocked by ethoxzolamide, further implicating the dependence of the anapleurotic reaction mediated by pyruvate carboxylase on CA VA.

As mentioned, carbon fixation at pyruvate carboxylase increases the concentration of mitochondrial intermediates for other biosynthetic reactions. For gluconeogenesis, it is malate that is drawn from the cycle. For lipogenesis, it is citrate that is drawn off the cycle. Citrate is made from the condensation of acetyl

CoA and oxaloacetate, the product of the pyruvate carboxylase reaction. Citrate can be transported out of the mitochondria where it is cleaved to re-form oxaloacetate and acetyl CoA, the latter of which is the substrate for cytoplasmic acetyl CoA carboxylase, the rate-limiting step in de novo lipogenesis. Hazen et al. showed that ethoxzolamide inhibits lipogenesis from pyruvate in 3T3-L1 adipocytes, a mouse adipocyte model [102]. Acetyl CoA carboxylase, like pyruvate carboxylase, utilizes HCO_3^- as a substrate, in this case for the carboxylation of acetyl CoA. That acetyl CoA carboxylase was not the target of sulfonamide inhibition was demonstrated by lack of sulfonamide inhibition of lipogenesis from glutamate, another anapleurotic substrate that increases the concentrations of Krebs cycle intermediates but independently from pyruvate carboxylation. ^{13}C-NMR studies, reported in 2009, support these conclusions [103]. Together, these data suggest that carboxylation of pyruvate by CA VB in the mitochondria of adipocytes is required for lipogenesis and by extension CA VA in liver mitochondria [104].

While mitochondrial diseases are often associated with defects in the oxidative phosphorylation [105], the above data suggest the possibility that the mitochondrial CAs could serve as targets for modulating gluconeogenesis and lipogenesis, both of which are dysregulated in obesity and insulin resistance. Interestingly, an adverse effect of sulfonamide- and sulfamate-containing anti-epileptic drugs is weight loss in obese patients [106]. Indeed, a randomized trial in 2003 demonstrated significant weight loss in a study of 60 non-epileptic obese patients given Zonisamide, a marketed anti-epileptic aliphatic sulfonamide with known serotonergic and dopaminergic activity in addition to blocking sodium and calcium channels [107]. Furthermore, Topiramate, a sulfamate-substituted saccharide, was approved for weight loss by the FDA in 2012 to be used in conjunction with phentermine treatment (which decreases appetite). While the mechanism for this effect is currently unknown, De Simone et al. have shown that Zonisamide strongly inhibits recombinant CA VA ($K_i = 20$ nM) [108]. Like Zonisamide, Topiramate inhibits CA VA, although with somewhat less efficacy ($K_i = 63$ nM) [13]. As an aside, Topiramate is also a strong inhibitor of CA VB (Ki = 30 nM), unlike Zonisamide which is relatively poor inhibitor ($K_i = 6.0$ μM). However, both drugs block CA II in low nM range which raises questions regarding the in vivo target. That said, Topiramate has been shown to be block lipogenesis from pyruvate, not acetate, in 3T3-L1 adipocytes [103]. Presently, each of these isoforms is being pursued as novel anti-obesity targets [109–111].

4 Secreted CAs

CA VI is the only secreted isoform among the α-carbonic anhydrase family [reviewed in [112]]. The existence of CA activity in saliva has been known for decades, but it was not until 1979 when it was realized that the activity was unique from that of erythrocyte CA activity (CAII) [113]. Feldstein and Silverman provided the initial biochemical and kinetic characterization revealing that rat salivary CA

VI had a molecular weight of 42 kDa and was glycosylated which predicted a secretory protein [114]. While the kinetic parameters were similar to that of CAII, CA VI exhibited a somewhat lower affinity for sulfonamide inhibitors. Murakami and Sly reported comparable data for CA isolated from human saliva, at which point the name CA VI was adopted [115]. Interestingly Parkkila et al. have shown that salivary CA VI secretion follows circadian rhythm [116], low during sleep and rising in concentration at awakening and breakfast. Subsequently, CA VI has been found in milk [117], tears [118], respiratory airways [119], epithelial lining of the alimentary canal [120], and enamel organs [121]. It has also been found in human serum [122]. Although the physiological function of CA VI is not fully established, there are clues that it regulates against acidic environments.

Saliva plays a critical role in oral homeostasis and decreased rates of secretion increases the risk of oral infections and dental caries [123]. The buffering capacity of salivary secretions depends primarily on bicarbonate ions and provides protection against enamel erosion [124]. Several studies have shown that CA VI is responsible for acid neutralization in dental biofilm, originating from bacterial metabolism. For example, Kimoto et al. showed that patients who rinsed their mouths with sucrose in the presence of acetazolamide had significantly higher salivary pH values than patients who rinsed with only sucrose [125]. In this study, CA activity associated with plaque was specifically identified as CA VI, not CA I or CA II. In another study, Kivela et al. demonstrated that a low concentration of CAVI in saliva is associated with a higher incidence of dental caries [126]. However, a study by Frasseto et al. revealed that CA VI activity in the oral cavity of children with dental caries was higher than that found in children who were caries-free, although the statistical significance of this observation was border-line [4]. Additionally, the variation in CA VI activity in saliva, before and after a sucrose wash, was significantly greater in children with dental caries than those without. Given that there did not appear to be differences in the concentration of CA VI, the authors suggest that genetic polymorphisms may be related to the differences in CA VI activity seen across these two patient populations. Indeed, polypmorphisms have been described that are associated with higher buffering activity but, interestingly, buffering capacity is decreased in healthy children [127]. These authors have suggested that polymorphisms in the coding region may affect secondary structure to alter CA VI function. Others have shown that both pH and buffering capacity of saliva is lower in diabetics compared to normal controls [128]. While CA activity was positively correlated with frequency of polymorphisms, there was no correlation between polymorphism frequency and pH or buffering capacity. These data suggest that there is still no consensus regarding the role of CA VI and pH control in the oral cavity.

CA VI is also known as gustin [129]. It has been shown that gustin is decreased in parotid saliva of patients who experience loss of taste [130–132]. This phenomenon was associated with aberrant taste bud morphology [133], consistent with apoptosis. Return of taste function has been demonstrated by exposure to exogenous zinc [132]. Interestingly, Topiramate and other CA sulfonamide inhibitors cause taste

perversion [134] perhaps targeting CA VI. Together, these data suggest a role for CA VI in taste perception. Perhaps weight loss in patients given Topiramate (see above) is in part related to the loss of food appreciation!

The crystal structure of CA VI has been solved revealing a prototypical mammalian CA fold, but with a novel dimeric arrangement as compared to previously reported CA structures [135]. The active site cavity contains a cluster of non-conserved residues that may be involved in ligand binding. This discovery may open opportunities for developing an isoform-specific inhibitor, which has been difficult because of the conservation in the catalytic site across most CA isoforms.

5 Membrane-Associated CAs

The human membrane-associated CAs include CA IV, CAIX, CA XII, and CA XIV. CA XV, like CA IV, is a GPI-anchored form of CA but is not expressed in humans or chimpanzees [136]. These enzymes are poised to reversibly hydrate CO_2 in the extracellular space. Several of these family members will be discussed in depth in later chapters in this book (Sly and Waheed, Chap. 9; Oosterwijk, Chap. 10; Benej et al., Chap. 11; Tafreshi et al., Chap. 12; and McDonald and Dedhar, Chap. 13), so please refer to those chapters as well.

A membrane-bound form of CA was initially purified from lung and tentatively called CA IV [137]. A "second" membrane-bound form was ultimately purified from human kidney [138]. Subsequently, Zhu and Sly reported a more efficient purification that allowed them to show that lung and kidney expressed the same membrane-bound form of CA [139]. These authors also showed that about 50 % of the enzyme could be released from the membrane by treatment with phosphoinositide-specific phospholipase C, suggesting that the enzyme is attached to the membrane by a GPI linkage. Human CA IV was cloned in 1992 by Okuyama et al. [140]. The deduced amino acids included an 18-amino acid signal sequence, a 260 amino acid stretch that show similarity to the catalytic regions of CA I, CA II, and CA III, with an additional 27 amino acid C-terminus containing a hydrophobic domain in the last 21 amino acids. Expression of CA IV cDNA in COS cells generated a 35 kDa membrane-bound protein. Baird et al. reported that CA IV is a high-activity isozyme showing pH independence in the hydration direction [141]. In the dehydration direction, the catalytic rate is even higher than that observed in CA II, although the esterase activity is lower. CA IV has also been localized to heart [142], brain [143], capillary bed of the eye [144], and erythrocytes [145].

Because kidney expresses both CA II and CA IV, the question arose as to whether the cytosolic CA or the membrane-bound CA was responsible for the CO_2 hydration that leads to the acidification of urine and reabsorption of filtered bicarbonate. In 1996, Conroy et al. designed a pegylated sulfonamide (F-3500) that inhibited CA activity, but was impermeant to cells [146]. This allowed the investigators to distinguish between intracellular and membrane-associated CA activity, and

specifically that of CA II and CA IV in kidney [147]. Low molecular weight CA inhibitors, like acetazolamide, produce urine with a concentration of 100–200 mM HCO_3^- in all mammalian species tested [148]. Under these circumstances, both intracellular and membrane-associated CA activity will be inhibited. In contrast, rats treated with F-3500 produced urine containing only 40 mM HCO_3^- that is taken as the effect of inhibiting CA IV while retaining CA II activity. These data support the hypothesis that both CA II and CA IV are important in bicarbonate reabsorption. These results agree with studies in humans lacking CA II where bicarbonate concentration became elevated in response to acetazolamide [149]. We now know that another membrane-associated CA (CA XII) is expressed in kidney [150, 151]. At this point in time, we cannot distinguish between CA IV and CA XII function for lack of isoform-specific inhibitors so the studies above could support the involvement of both CA IV and CA XII in bicarbonate reabsorption in the kidney.

Like CA II, CA IV interacts with Cl^-/HCO_3^- transporters [152]. In this study, Sterling et al. demonstrated that CA IV interacts directly with the 4th extracellular loop of AE1. This interaction increases the activity of bicarbonate transport. CA IV also creates a functional complex with the Na^+/bicarbonate co-transporter (NBC1) [153]. This latter study showed that this interaction is required for maintaining appropriate pH balance within the environment of the retina and retinal pigment epithelium, although neither CA IV nor NBC1 are expressed in the retinal or retinal pigment epithelium. This requirement is based on the finding that mutant forms of CA IV appear to be responsible for an autosomal dominant form of retinitis pigmentosa [154] causing rod and cone photoreceptor degeneration [153]. These mutations are associated with a loss of CA activity or the inability of CA IV to interact with NCB1, in choriocapillaris leading to impaired pH homeostasis [153]. Based on the importance of CA IV in the survival of photoreceptor cells, this raises a flag for long-term use of CA inhibitors, particularly in the treatment of glaucoma, which may adversely affect vision.

CA IX and CA XII are specifically tumor-related [14, 155]. CA IX has garnered more interest because of its limited normal expression [156, 157], and its apparent role in cell proliferation and migration [158, 159], cell adhesion [160], tumorigenesis [161], and pH control [162–165]. CA IX is a transmembrane glycoprotein whose catalytic domain is oriented toward the extracellular milieu [166]. CAIX is expressed as a 49.7 kDa protein but is truncated to the mature form during processing [167]. This mature form contains an N-terminal “exofacial” proteoglycan-like domain and catalytic domain (homologous to CA II) that is attached via a transmembrane segment to a cytoplasmic tail. CA IX exists primarily as a dimer stabilized by disulfide bonds [168–170]. In rat cardiomyocytes, CA IX interacts with the NBCe1 Na^+/HCO_3^- cotransporter enhancing bicarbonate influx [171]. The cytoplasmic tail of CA IX contains the phosphorylation motif for protein kinase A (PKA) that is important for catalytic activity [15]. Recombinant CA IX, containing the catalytic domain has activity similar to that of CA II [168, 172]. CA IX is regulated by hypoxia [173], and in general predicts poor patient

outcome [174–177]. The secondary structure and orientation of CA XII is similar to that of CA IX, but is a monomer, lacks the proteoglycan-like domain, and is missing the PKA motif [150]. Its catalytic activity is lower than that of CA IX [13, 178, 179] which may influence its role in pH control in tissues compared to CA IX. Northern blot analysis initially revealed CA XII expression in kidney and colon [150]. Western blotting and immunohistochemistry show a much wider tissue distribution including kidney, lung, prostate, ovaries, uterine endometrium, breast, and the basolateral membrane of epithelial cells of the gut [155, 180–182]. In contrast to CA IX, CA XII is regulated by estrogen [183, 184]. In breast cancer patients, CA XII expression correlates with positive prognosis [183–186]. In other cancers, CA XII expression can be positive, negative, or neutral as a predictor of patient outcome [177, 182, 187, 188]. Interestingly, CA XII has been associated with metabolic acidosis in patients receiving carbonic anhydrase inhibitors, specifically Topiramate or Zonisamide (see Sects. 3 and 4, this chapter) [189]. Patients sensitive to these drugs typically have serum bicarbonate concentrations of less than 20 mM. Low bicarbonate was associated with polymorphisms in CA XII (rs2306719 and rs4984241). While these data warrant further investigation, this indicates a role of CA XII in renal function. In addition, a Glu143Lys mutation in CA XII has been linked to individuals with failure to thrive, hypoatremic dehydration and hyperkalemica with isolated sweat salt wasting [190]. This autosomal recessive mutation behaves similarly to the excessive salt loss from sweat glands observed in pseudohypoaldosteronism type 1 which arises from mutations in genes encoding epithelial Na^+ channel (ENaC) subunits. These data demonstrate the importance of bicarbonate anion and proton production on salt concentration in sweat and its significance for sodium homeostasis, and implies a specific role for CA XII.

The least studied of the human CAs is CA XIV which was cloned in 1999 [191] and bears strongest sequence similarity to CA XII. CA XIV mRNA shows strong expression in most parts of the brain with weaker signals in colon, small intestine, urinary bladder and kidney. RT-PCR analysis revealed an intense signal in liver and spinal cord, but much weaker in kidney. However, by western blot and immunohistochemistry, CA XIV shows significant luminal co-localization with CA IV (but not CA XII) in regions that are involved with urinary acidification [192]. This suggests functional overlap between CA IV and CA XIV. CA XIV has also been implicated in acid–base balance in muscle and erythrocytes in an adaptive response to chronic hypoxia as observed at high elevation [193]. Like other membrane-bound CAs, CA XIV interacts with bicarbonate transporters [194]. In heart myocardium, it has been demonstrated that CA XIV interacts with AE3. In hypertrophic hearts from hypertensive rats, CA XIV expression is elevated along with AE-mediated bicarbonate transporter. This suggests a role for CA XIV in AE3 hyperactivity. Finally, CA XIV, in contrast to CA IV, has been localized to the apical and basal membranes of the retinal pigment epithelium, along with the plasma membrane of Müller cells [195]. Because CA II is also found in Müller cells [196], this implies that CA II and CA XIV have specific and unique functions in the context of acid based balance in the retina.

6 Concluding Remarks

While the physiological functions of some of the mammalian isozymes of CA are still uncertain, it is clear that the CAs are important in many physiological processes in both normal and pathological states. CA inhibitors are now widely used in the clinic to treat a number of diseases including glaucoma, epilepsy, mountain sickness, ulcers, osteoporosis, and obesity. Yet, these inhibitors collectively target enzymatic activity, which limits their targeting specificity because of the structural similarity of the CA catalytic pockets. Thus, our greatest challenge is to develop CA-specific inhibitors to further our understanding of function, develop diagnostic tools, and treat diseases in a selective fashion. This requires a better understanding of the CA structures to facilitate the design of novel drugs. In addition, it may be possible to use surface motifs for docking a catalytic inhibitor to provide specificity. Also encouraging are the membrane-impermeant compounds, which block only membrane-associated CA isoforms (discussed in Chap. 15, McKenna and Supuran). These should increase our ability to target cancer-related CAs, like CA IX and CA XII. One can also imagine nanoparticle delivery systems that use cell surface epitopes for tissue-specific drug targeting. While we have a long road ahead in the discovery process, it is clear that the stakes are high in exploiting the secrets of this ancient but critical enzyme.

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