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Chemical Properties of Carbonic Anhydrase IV, the Membrane-Bound Enzyme

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SUMMARY

The carbonic anhydrase (CA) isozyme (IV) in microsomes is thought to have a dominant role in secretory processes. Using microsomes from bovine kidney and lung (which had the same activity), we have measured the K_m and k_{cat} for CO_2 hydration and compared these numbers with those for CA I (red blood cells and gut), CA II (red blood cells and secretory cells), and CA III (muscle). For kidney CA IV, K_m is 10 mM and k_{cat} is 170,000 sec^{-1} at 0°, approaching the rate for CA II but much greater than those for CA I or III. The K_i values for 11 sulfonamides with CA IV were measured and in all cases showed less binding (averaging 17-fold) than to CA II. This is the result of reduction of the association rate constants (k_{on}), whereas the dissociation constants of the drug-enzyme complexes (k_{off}) are similar between CA II and IV. Based on these data, full physiological effects may be expected when inhibition of CA IV is about 99%. Anion inhibition of CA IV is similar to that of CA II and less than that of CA I or CA III. Data are compatible with the proposed role of CA IV in physiological events, i.e., HCO_3^- formation and secretion at one cell border and H^+ separation and excretion at the other.

We recognized many years ago that CA, earlier held to be a cytosolic enzyme, was also present in microsomes and mitochondria of rat kidney and liver (1, 2). Since that time, these enzymes have been given full status as CA IV and CA V, respectively. The membrane-bound enzyme (CA IV) from kidney and lung of rats, rabbits, dogs, and humans has been studied and its properties reviewed (3–7). CA IV has been found as well in all other secretory tissues in which it has been sought, including colon, pancreas, and brain (8, 9).[1] There are at least five characteristics of CA IV that distinguish it from CA II, the cytosolic enzyme. 1) CA IV is less susceptible to sulfonamide inhibition, by approximately 15-fold. 2) CA IV is resistant to denaturation by sodium dodecyl sulfate. 3) CA IV is partially anchored to renal apical membranes by phosphatidylinositol glycan linkage. 4) In cows, the molecular weight is about 52,000, due to attachment of five or six *N*-linked oligosaccharide chains. This is not the case for human lung or kidney membranes, whose CA has a molecular weight of 35,000. Despite this, the activity and the susceptibility to inhibition of the bovine and human enzymes appear to be the same. 5) CA IV and CA II are antigenically distinct and cDNA has been isolated, analyzed, and expressed. The catalytic activity of CA IV has been found to be similar to that of CA II. Kidney and lung enzymes appear to be structurally identical. Membrane-bound CA from rabbit ciliary processes also appears similar (10). This is important in view of the action of inhibitors on secretion from this tissue and their use in the treatment of glaucoma (11).

In the present work we 1) measure the kinetic properties (k_{cat} and K_m) of CA IV, comparing them with those of CA I-III, 2) measure the inhibition constants (K_i) of 11 sulfonamides and three anions of varying structural and physicochemical properties, including five new compounds, again comparing the four isozymes, and 3) measure rate constants of four of the compounds with the enzymes, comparing CA IV and II. On the basis of this work we reevaluate the relation between enzyme inhibition and pharmacological response.

It is likely that this isozyme is of great importance in transport processes, because the secretory cells have vectorial properties, i.e., HCO_3^- or H^+ secretion is directed toward basal or apical surfaces (11, 12). Significantly, we have shown that, in mice lacking the cytosolic (II) enzyme, renal HCO_3^- reabsorption and the response to specific inhibitors of CA are normal (13). Thus, CA IV appears to be the dominant isozyme in renal secretion. Washed particulate fractions from homogenized pigmented and nonpigmented bovine ciliary epithelium show CA activity (14). Mice lacking cytosolic CA II show membrane

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[1] Exceptions are the reports that CA IV is absent in the ciliary process and choroid plexus, as studied by immunohistochemistry (9, 27). However, other evidence from chemical or inhibition analyses suggests strongly that membrane-bound enzyme is present in these tissues (see Refs. 14 and 15). Importantly, membrane-bound enzyme is found by cobalt staining in ciliary processes[2] and choroid plexus (P. J. Wistrand, personal communication) of CA II-deficient mice.

ABBREVIATION: CA, carbonic anhydrase.

staining in the eye just like normal mice.[2] There is also functional evidence for enzyme activity in the basolateral membranes of nonpigmented epithelium in ciliary processes (15). We believe that membrane-bound enzyme (CA IV) is of universal importance in the secretion of H^+ or HCO_3^- ions and the subsequent movement of fluid.

Materials and Methods

Analytical methods and kinetic assays. The reaction rates and inhibition constants were measured by methods previously described in this laboratory (16, 17). For the determination of V_{max} and K_m, substrate was varied by the use of tanks of CO_2 at 8, 16, 50, and 100%, corresponding to 5.6, 11.2, 35, and 70 mM, respectively. All reactions were done at 0°. CO_2 was bubbled into a reaction vessel containing a solution of bromthymol blue or phenol red. For the uncatalyzed rate the reaction was then started by addition of barbital buffer at its pK_a, 7.9. The time to reach pH 7.2, which titrates 85% of the buffer, was measured by electrode or color change.

For determination of catalyzed or catalyzed-inhibited rates, enzyme or enzyme-drug mixtures were added and equilibrated (2–5 min) with the indicator solution. For measurement of catalytic rates, as shown in the Lineweaver-Burk plots, the uncatalyzed rates (see below) were subtracted from the observed rates. The reactions represent titration of essentially all of the sodium barbital present (7 mM) and require 36 sec for 100% CO_2 in the absence of enzyme. This uncatalyzed rate is then 195 μM/sec, with one half, one sixth, and one twelfth of this value for the successively lower concentrations of CO_2 listed above. The rate constant (k_{CO_2}) is then 0.0028 sec^{-1}, agreeing well with measurements by a variety of methods showing independence of k_{CO_2} from pH (18). When one enzyme unit is added, the rate is doubled (by definition), i.e., for 100% CO_2 the time is 18 sec. For various concentrations of drug the fractional inhibition (i) is calculated, yielding the K_i (17). The methods for obtaining the association (k_{on}) and dissociation (k_{off}) rate constants are also given in Ref. 18. The enzymic rate constant k_{cat} (see Results and Table 1) for CA II agrees well with that obtained using a stopped-flow constant pH system at 5° and calculated for enzymic pK_a of 7.5 (19).

The molar concentrations of enzyme (E_o) were obtained from titration of enzyme with powerful inhibitors, as shown in Fig. 1 for chlorzolamide. k_{cat} was then calculated from V_{max} in Lineweaver-Burk plots, which also yield K_m.

All determinations were replicated at least 10 times. The standard errors were approximately ±20% of the means. Sets of numbers differing by <2-fold are not considered significantly different.

Structures and sources of drugs are given in Ref. 18, with additions as noted in Table 2.

Enzyme sources and preparation. This follows broadly the published method for purification of CA IV from human lung and kidney (4, 5). Calf kidneys were obtained from a slaughterhouse and were frozen at −79° or used at once. They were cut into small pieces and homogenized for 6 min in a blender with 2 volumes of buffer mixture containing 25 mM triethanolamine sulfate, 50 mM sodium sulfate, and 1 mM benzamidine. The homogenate was filtered through cheesecloth, and the filtrate was centrifuged at 20,000 × g for 30 min. The supernate was then centrifuged at 235,000 × g for 1 hr. The pellet containing microsomes and plasma membranes was suspended in the buffer and centrifuged. The washing procedure was repeated three times. There was no CA activity in the supernate at the end of the process. CA activity was determined in the washed membrane fractions. This material was then suspended in a small volume of buffer and used for the inhibition and kinetic analyses. The protein concentration of these membranes was 0.49 g/g of packed pellet, determined according to the method of Lowry *et al.* These membranes contained 80 enzyme units (as defined above)/g for kidney and 40 units/g for lung.

CA I was a gift from Dr. Harvey Schwam, Merck Sharp & Dohme. It was purified from human red blood cells by affinity chromatography. CA II (bovine) was purchased from Sigma Chemical Co. (St. Louis, MO) or obtained from dog red blood cell hemolysate (20). CA III was prepared from bovine muscle; the microsomes were separated by differential centrifugation and used directly in the assays.

Results

Fig. 1 shows an Easson-Stedman plot for inhibition of CA IV from bovine kidney. The derivation has been given (20), as well as the application to membrane-bound CA (1, 3). The data yield the K_i for the inhibitor (slope) as well as the important value of E_o (ordinal intercept), the enzyme concentration that is used in the kinetic and inhibition data. In the present case, K_i for chlorzolamide is 1.9×10^{-8} M and the ordinal intercept E_o is 2×10^{-9} M. Because 1.7 enzyme units were used, E_o for 1 enzyme unit is 1.2×10^{-9} M.

Fig. 2 shows a Lineweaver-Burk plot for CA IV from bovine kidney (Fig. 2A) and lung (Fig. 2B). From these data and Fig. 1, we obtain K_m and k_{cat} as follows. From the abscissal intercepts of Fig. 2, K_m is observed at 10 mM for kidney and 8 mM for lung. V_{max} for kidney (Fig. 2A) is 357×10^{-6} M/sec; 1.75 enzyme units were used, or 2.1×10^{-9} M (see Fig. 1).

$$k_{cat} = \frac{V_{max}}{E_o} = \frac{347 \times 10^{-6}\ \text{M/sec}}{2.1 \times 10^{-9}\ \text{M}} = 1.7 \times 10^5\ \text{sec}^{-1}$$

Similar calculations for lung (Fig. 2B), where 1.33 enzyme units were used (1.6×10^{-9} M), show V_{max} at 250×10^{-6} M/sec, yielding k_{cat} of 1.6×10^5 sec^{-1}.

Table 1 gives comparative kinetic data for CA I-IV. It is immediately apparent that CA IV closely resembles II. CA I has (in terms of k_{cat}/K_m) about 28% of the activity of II,[3] but III has only 0.1% of the activity of II. The differences are in no way species related. The isozymes are kinetically alike in the different species; membrane and cytosolic enzymes have been compared in the same species (1–4, 6, 10, 13).

Table 2 gives the inhibition data for CA IV, comparing them with those for CA I, II, and III. The chief interest is comparison

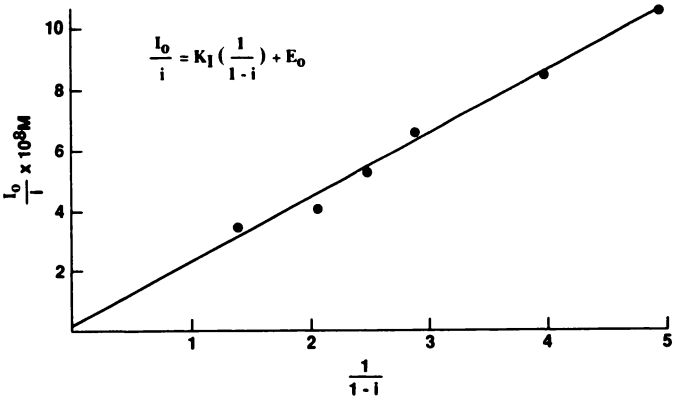

Fig. 1. Easson-Stedman plot of chlorzolamide against bovine kidney microsomes (1.7 enzyme units) with 70 mM CO_2 hydration, at 0°, in barbital buffer. E_o (ordinal intercept) = 2×10^{-9} M, K_I (slope) = 1.9×10^{-8} M.

[2] Y. Ridderstråle, P. J. Wistrand, and W. F. Brechue. Membrane-associated carbonic anhydrase activity in the eye of CA II-deficient mice. Submitted for publication.

[3] However, this is diminished to about 15% *in vivo* because of chloride inhibition of CA I (28).

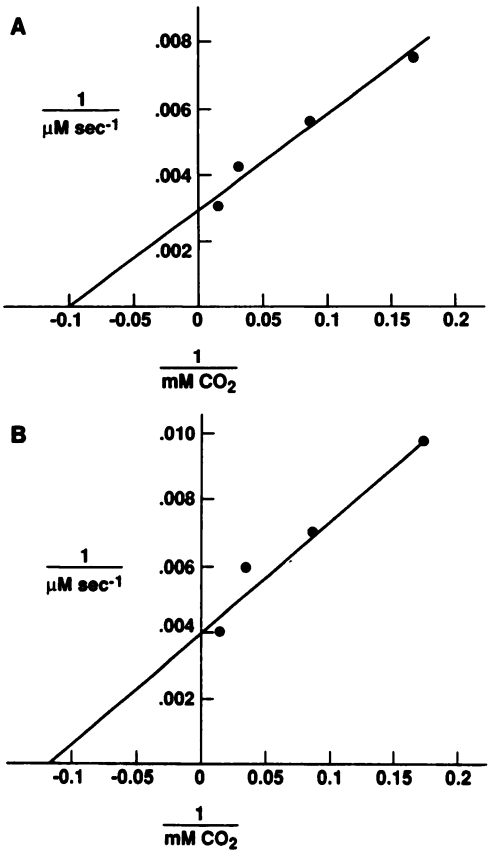

Fig. 2. Lineweaver-Burk plots of CA IV from bovine kidney (A) and lung (B). Conditions were as in Fig. 1. A, $E_o = 2.1 \times 10^{-9}$ M, $k_{cat} = 1.7 \times 10^5$ sec^{-1}, $K_m = 10$ mM. B, $E_o = 1.6 \times 10^{-9}$ M, $k_{cat} = 1.6 \times 10^5$ sec^{-1}, $K_m = 8$ mM.

TABLE 1

Kinetics of CA IV, compared with other isozymes

Data were obtained at 0–1°

	I[a]	II[a]	III[b]	IV[c]	IV[d]
K_m (mM)	4.4	10	40	10	8
k_{cat} (sec^{-1})	29,000	236,000	1,000	170,000	160,000

[a] Human red blood cells (30).
[b] Cat muscle (30).
[c] Bovine kidney (present work).
[d] Bovine lung (present work).

between II and IV. All 11 sulfonamides are less inhibitory against IV than II, with ratios of K_i values ranging from 7 to 33 and centering at about 17. This relationship appears to be independent of the type of sulfonamide structure or physicochemical properties; these are given in Refs. 17 and 21. In contrast, the anions do not discriminate between II and IV but are far more inhibitory (and equally so) against I ≃ III than against II ≃ IV.

It may be asked whether the differences in K_i values between CA IV and II are due to nonspecific binding to membrane lipids or other constituents of the cell for CA IV. This is answered by the fact that a weak inhibitor, sulfanilamide, shows the same ratio of inhibition constants (CA IV/II) as do compounds that are thousands of times more active. Clearly, if there was nonspecific binding, the distortion would be much greater for the compounds used in lower concentrations. Furthermore, the ratios do not vary among lipid-soluble (i.e., ethoxzolamide) and

TABLE 2

Inhibition by sulfonamides and anions of CA IV, in comparison with isozymes I–III

Structures, properties, and sources are given in Ref. 17. Data were obtained at 0°, with enzyme-inhibitor equilibration.

Compound	K_i			
	IV	II	I	III
	nM			
Sulfanilamide	26,000	1,000	50,000	$>5 \times 10^6$
AHR-16329[a]	330	10		6×10^4
Chlorthiazide	110	16		
Acetazolamide	66	7	200	3×10^5
Methazolamide	56	8	10	1×10^5
MK-417[b]	33 (7)[c]	2 (0.3)[c]	$>5 \times 10^6$	8×10^4
CL-13,475[d]	70	3		3×10^3
Benzolamide	20	1	2	3×10^3
Chlorzolamide	19	1	1	300
Ethoxzolamide	13	0.7	1	5×10^4
L-662,583[e]	10	0.4	10,000	
Cl^-	5×10^8	2×10^8	6×10^6	6×10^6
I^-	3×10^7	3×10^7	3×10^5	1×10^6
CNO^-	3×10^4	2×10^4	7×10^2	5×10^2

[a] Ref. 31.
[b] This is the *S*-(+)-enantiomer of MK-927 (17).
[c] The values in parentheses are obtained when MK-417 or MK-927 is preequilibrated with enzyme at 37° and the reaction is then run at 0°. See Ref. 32. This odd phenomenon is observed only in the thieno-thiopyran series. We are grateful to Dr. Harvey Schwam at Merck Sharp & Dohme Research Laboratories for calling this to our attention.
[d] 2-*p*-Aminobenzenesulfonamido-1,3,4-thiadiazole-5-sulfonamide (American Cyanamid).
[e] Ref. 21.

TABLE 3

Rate constants for representative sulfonamides against CA IV, compared with CA II

	K_i (× 10^9)	k_{on}	k_{off}
	M	$L/mol\ sec^{-1}$	sec^{-1}
Sulfanilamide			
IV	26,000	1.1×10^3	0.029
II	1,000	1.3×10^4	0.013
Methazolamide			
IV	56	3.6×10^5	0.020
II	8	3.0×10^6	0.024
MK-417			
IV	33	3.6×10^5	0.012
II	2	7.5×10^6	0.015
Ethoxzolamide			
IV	13	1.9×10^6	0.025
II	0.7	26×10^6	0.018
Mean ratio, IV/II	17	0.08	1.3

lipid-insoluble (i.e., benzolamide) compounds of the highly active class, precluding the possibility of partitioning into non-CA structures.

Table 3 shows k_{on} and k_{off} constants for four of the compounds with CA IV, comparing them with the reactions with CA II. [It should be noted that these data are for 0° and 70 mM CO_2 and so differ slightly but significantly from numbers obtained for CA II at 25° and 6 mM CO_2 (17).] Table 3 shows clearly that the lower activity of the compounds against CA IV, compared with CA II, lies in the slower k_{on}. The ratios of IV/II for the k_{on} range from 0.05 to 0.12 (mean, 0.08), whereas for k_{off} they are close to unity (range, 0.8–2.2; mean, 1.3).

Discussion

Our principal findings are that the hydration kinetics of the membrane-bound CA IV from kidney and lung are similar to

those of CA II, with K_m being approximately 10 mM and k_{cat} approximately 2×10^5 sec^{-1} at 0°. Inhibition by sulfonamides, however, is weaker (by 7–33-fold) for CA IV than CA II for all 11 compounds studied. Anion sensitivity is the same for II and IV. We also show comparative kinetic and inhibition data for CA I and CA III. In the absence of X-ray crystallographic data on the active site, there is no structural explanation for the lesser sulfonamide inhibition of CA IV. It is clear that this lesser activity is due to slower association rates between sulfonamide and enzyme, with the dissociation rates being about the same for drugs with the two enzymes. This mirrors our previous finding (17) (also evident in Table 3) that drug potencies against the same enzyme are a function of k_{on}. This appears reasonable, because widely differing drug structures would be expected to have differing access to the active site of the enzyme, based on hydrophobic interactions, ionization of sulfonamide, and binding to zinc. Once bound, however, their dissociations should be similar, because the critical step in drug dissociation is the breaking of the strong zinc-sulfonamide bond, which is similar among the various compounds. Notably, the k_{off} values for anions are orders of magnitude greater and differ greatly among themselves (17).

These findings have several consequences. The first is the confirmation that a powerful CA exists in membranes as well as in cytosol, overturning the old idea that CA is basically a cytosolic enzyme. In retrospect, it is curious that an enzyme associated with the vectorial movement of H^+ or HCO_3^- was once thought to be restricted to cell fluid. Now the question may be reversed; what is the role of CA II? This is emphasized by our finding that mice lacking CA II have essentially the classic urinary electrolyte response to sulfonamide inhibitors (13).

A second consequence is a reevaluation of a problem we posed 30 years ago, i.e., the relation in the CA system between enzyme activity or enzyme inhibition and physiological rates or pharmacological response. There were and are two independent approaches. The first was a comparison of enzymic and nonenzymic rates with physiological rates, i.e., rates of HCO_3^- secretion in aqueous humor of eye, cerebrospinal fluid, or pancreas or H^+ secretion in kidney or stomach (12, 22). The second was the calculation of fractional inhibition (i) of CA (at a time when CA IV was not recognized) in tissues after administration of sulfonamide drugs. This was possible because we knew the concentration of drug and original enzyme in the tissue and the K_i of the drug-enzyme complex (12, 23).

The first of these approaches yielded the result that the observed rates *in vivo* (V_{obs}) were 2–3 times greater than calculated uncatalyzed rates (V_{unc}) but approximately 0.1–1% of the calculated enzymic reaction rates (V_{enz}). The calculations for V_{unc} and V_{enz} included several approximations, or (more frankly put) guesses, notably regarding the pH and the volume of the secretory site. It did seem clear, however, that V_{enz}/V_{unc} was very high, approximately 10^3 to 10^4, agreeing with *in vitro* measurements showing that CA II (cytosolic) is an exceptionally active enzyme, with the highest turnover number known (1.3×10^6 sec^{-1} at 25°) (see also Table 1). The relation V_{enz}/V_{obs} was also very high, suggesting a great excess (100–1000-fold) of enzyme over physiological needs (12).

The second approach, calculation of i, the fractional inhibition (Table 4), from I_o (tissue concentration of drug), E_o (CA II concentration in whole cells), and K_i (dissociation constant of the enzyme-inhibitor complex), yielded the surprising result that, when i was 0.990, there was no pharmacological effect. The dose-response curve began at about 0.993, and maximal effects were observed at about 0.999. There were minor differences in these values among the different organ systems, depending on differing values for E_o. In these analyses care was taken to use diffusible drugs so that I_o was a function of unbound plasma concentration or free drug in the tissues (23).

TABLE 4

Fractional inhibition of CA II and CA IV after topical (corneal) or intravenous administration of sulfonamides

Compound and time for full effect on intraocular pressure (24, 33)	Concentration, I_{free}	K_i[a]		i[b]	
		II	IV	II	IV
	μM	nM			
MK-417, 1 drop of 0.5% solution to cornea, 2 hr	1.8[c]	1	21	0.9994	0.989
Methazolamide, 4 mg/kg intravenously, 0.5 hr	14[d]	24	168	0.9983	0.986

[a] From Table 2, adjusted to 37°, by 3-fold increase in K_i from 0° value (C.W. Conroy and T. H. Maren, Thermodynamics of sulfonamide binding to human carbonic anhydrases I and II, manuscript in preparation).

[b] $i = I_{free}/(I_{free} + K_i)$ (22, 23).

[c] From measured drug concentration in ciliary process (I_0) − drug bound to enzyme (EI); EI in ciliary process is taken as $\simeq E_0 = 0.8$ μM (34).

[d] From plasma concentration × unbound fraction.

The results of these two independent approaches agreed, in that approximately 99.9% of the enzyme must be inhibited to produce a pharmacological effect. It must be emphasized that the second approach is more rigorous and involves no assumptions about cell structure, volume, or pH. It is this second approach that must now be modified, because the K_i of the CA IV complex is approximately 18 times higher (on average, for the various drugs used; Table 2) than that for CA II, which was the basis of the original calculations. Thus, what we called 99.9% ($i = 0.999$) for CA II would become 98.2% ($i = 0.982$) for CA IV.

Table 4 shows specific examples of this, for topically applied MK-417 or MK-927 (21) and systemic methazolamide (31). We used the minimal doses (one drop of 0.5% solution and 4 mg/kg, respectively) for full effect in lowering intraocular pressure in rabbits. It is significant that i values for the two compounds, of very different structures and different routes of administration, agree well for each of the two enzymes. Although i for enzyme IV is less than that for II, it still is within about 1% of complete inhibition.

These data do not controvert the first approach described above, because calculations using different estimates for volumes or pH could yield ratios of V_{enz}/V_{obs} in the range of 50–200-fold, rather than the larger values ($>10^3$) cited above. These smaller ratios would also yield i of 0.98–0.995, in agreement with Table 4.

The problem regarding the relation between membrane-bound and cytosolic CA remains. Because they are different gene products, CA II cannot be regarded as a precursor of CA IV. The absence of CA II is clearly compatible with life, in mice (13) and humans (25).[4]

We may say tentatively that membrane-bound enzyme is

[4] The syndrome of osteopetrosis, renal tubular acidosis, and cerebral calcification is associated with CA II deficiency in humans (25). It is not, however, clear that these are all a direct result of the enzyme deficiency. Notably, CA II-deficient mice do not show all of these signs (13), nor do animals whose CA has been inhibited for most of their lives (29).

responsible for formation of HCO_3^- and thus a link to secretion of Na^+. In the special case of kidney, it also ensures equilibrium pH among the CO_2 species in fluid of the tubular lumen. Cytosolic enzyme keeps the CO_2 species at equilibrium within the cell or, in the important case of red blood cells, makes HCO_3^- and CO_2 rapidly interchangeable for carriage and release. CA IV, however, appears to be the major isozyme in ion movement and fluid secretion (12, 26).

Note added in Proof

The nucleotide sequence of the CA IV gene has now been published and assigned to chromosome 17q23, in contrast to CA I-III, all localized at chromosome 8q22 (35).

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