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# Carbonic anhydrase activators: An activation study of the human mitochondrial isoforms VA and VB with amino acids and amines

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**Abstract**—The mitochondrial isozymes of human carbonic anhydrase (hCA, EC 4.2.1.1), hCA VA and hCA VB, were investigated for activation with a series of amino acids and amines. D-His, L-DOPA, histamine, dopamine, and 4-(2-aminoethyl)morpholine were excellent hCA VA activators, with $K_A$s in the range of 10–130 nM. Good hCA VB activating effects were identified for L-His, D-Phe, D-DOPA, L-Trp, L-Tyr, serotonin, and 2-(2-aminoethyl)-pyridine, with $K_A$s in the range of 44–110 nM. All these activators enhanced $k_{cat}$, having no effect on $K_M$, favoring thus the rate-determining step in the catalytic cycle, the proton transfer reactions between the active site and environment. The activation pattern of the two mitochondrial isoforms is very different from each other and as compared to those of the cytosolic isoforms hCA I and II.


The carbonic anhydrases (CAs, EC 4.2.1.1) are ubiquitous metallo-enzymes, present in prokaryotes and eukaryotes, being encoded by four distinct, evolutionarily unrelated gene families: the α-CAs (present in vertebrates, Bacteria, algae, and cytoplasm of green plants), the β-CAs (predominantly in Bacteria, algae, and chloroplasts of both mono- as well as dicotyledons), the γ-CAs (mainly in Archaea and some Bacteria), and the δ-CAs, present in some marine diatoms, respectively.[1–8] In mammals, 16 different α-CA isozymes or CA-related proteins (CARP) were described, with very different subcellular localization and tissue distribution.[1–8] Basically, among the 13 catalytically active enzymes there are five cytosolic forms (CA I-III, CA VII, and CA XIII), five membrane-bound isozymes (CA IV, CA IX, CA XII, CA XIV, and CA XV), two mitochondrial forms (CA VA and CA VB), as well as a secreted CA isozyme (in saliva and milk), CA VI. Among the membrane-bound CAs, isoforms CA IV and XV are anchored to membranes by means of GPI (glycosylphosphatidylinositol) tails, whereas isozymes IX, XII, and XIV are transmembrane proteins possessing just one transmembrane domain. However, all these five isozymes have their active site outside the cell, being commonly termed as extracellular CAs.[1–8] Three of these proteins, that is, CARP VIII, X, and XI, are devoid of catalytic activity, as they lack at least one histidine residue among the three such residues that coordinate the catalytically crucial Zn(II) ion within the active site.

These enzymes catalyze a very simple physiological reaction, the interconversion between carbon dioxide and the bicarbonate ion, and are thus involved in crucial physiological processes connected with respiration and transport of $CO_2$/bicarbonate between metabolizing tissues and lungs, pH and $CO_2$ homeostasis, electrolyte secretion in a variety of tissues/organs, biosynthetic reactions (such as gluconeogenesis, lipogenesis, and ureagenesis), bone resorption, calcification, tumorigenicity, and many other physiologic or pathologic processes. Many of these isozymes are important targets for the design of inhibitors or activators with clinical applications.[1–8] CA inhibitors were much investigated and some compounds are clinically used as diuretics, antiglaucoma, anti-obesity or antitumor (diagnostic) agents.[1–8] On the contrary, CA activators (CAAs) are by far much less investigated.[7]

*Keywords*: Carbonic anhydrase; Isoform VA; VB; Activation; Amine; Amino acid; Histamine; Mitochondria.
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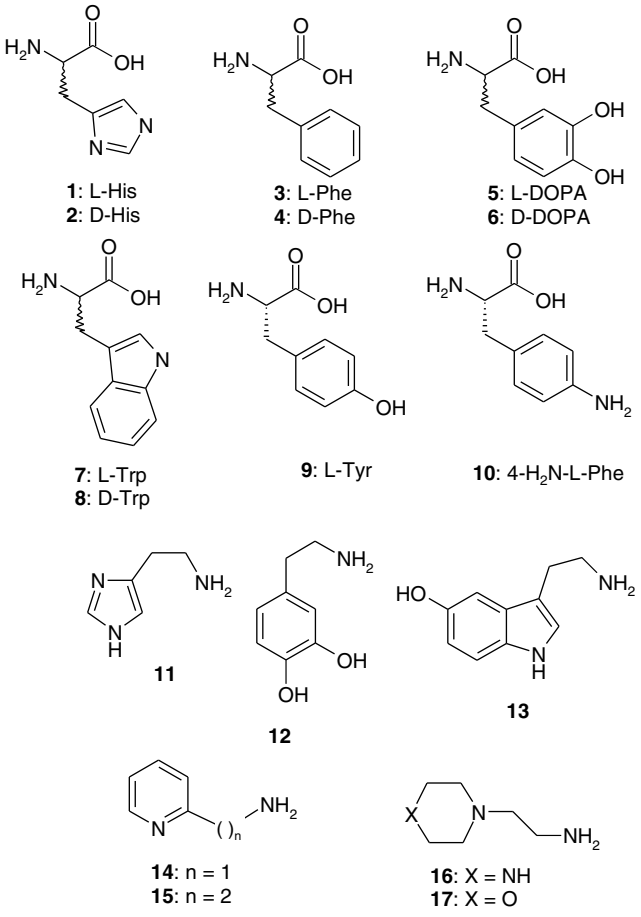
A multitude of physiologically relevant compounds such as amino acids, oligopeptides or small proteins, as well as many biogenic amines (among which histamine, serotonin, and catecholamines), were shown to efficiently activate the catalytic activity of several CA isozymes, such as CA I, II, and IV.[7,9,10] Activation of the cytosolic, ubiquitous isoforms CA I and II was then shown to constitute a possible therapy for the enhancement of synaptic efficacy, which may represent a conceptually new approach in the treatment of Alzheimer's disease, aging, and some other disease conditions characterized by an eventual loss of memory functions.[11,12] Recently, by means of electronic spectroscopy, X-ray crystallography, and kinetic measurements, it has been proved that CAAs bind within the enzyme active cavity (in the case of the physiologically most important isoforms, hCA I and hCA II) at a site distinct of the inhibitor or substrate-binding sites, participating thereafter in the rate-determining step of the catalytic cycle, the proton transfer reaction between the active site and the environment.[9,10,13–15] Previous data[7,10,13–15] on the activation of the cytosolic isoforms CA I, II, and XIII clearly showed that a CAA must possess specific steric and electronic requirements for good activity, that is, it must fit within the restricted active site cavity of the enzyme, favorably interacting with amino acid residues present in the activator binding pocket, and second, it should possess a moiety able to participate in proton transfer processes, better if with a p$K_a$ in the range of 6.0–8.0. No activation studies of the two mitochondrial isoforms hCA VA and hCA VB, which play important physiological roles in mitochondrial pH regulation and biosynthetic reactions among others,[16–19] have been reported up to now, except the activation constants of L-/D-Phe and L-/D-His against hCA VA in some of our previous work.[13,14] Thus, we decided to investigate a larger series of amino acid and amine derivatives (of types **1**–**17**) for their interaction with the two mitochondrial forms hCA VA and hCA VB.

L-/D-Amino acids and amines **1**–**17** investigated as hCA VA/B activators were commercially available from Sigma–Aldrich (Milan, Italy) and were used without further purification. The recombinant CA isozymes (hCA I, II, VA, and VB) have been obtained as described earlier.[16,17]

Kinetic experiments for the physiological reaction (carbon dioxide hydration to bicarbonate and a proton) (Table 1) showed that as for hCA I and II,[7,13–15] activators of the amino acid or amine type enhance $k_{cat}$ of the enzyme, with no effect on $K_M$. Indeed, as observed from data of Table 1, L- or D-Phe (compounds **3** and **4**) at a concentration of 10 μM produced a notable enhancement of $k_{cat}$ for all four investigated isoforms, that is, hCA I, hCA II, and hCA VA/hCA VB. Thus, for hCA I, this parameter for the pure enzyme is of $2.0 \times 10^5$ s$^{-1}$,[10] whereas in the presence of activators **3** and **4** at a concentration of 10 μM, it becomes of 2.3–$19.8 \times 10^5$ s$^{-1}$. For hCA II, the enhancement of $k_{cat}$ from the value of the pure enzyme ($1.4 \times 10^6$ s$^{-1}$) is in the range of 5.2–$5.7 \times 10^6$ s$^{-1}$ in the presence of the two activators. For hCA VA, this enhancement is of




4.3–$9.7 \times 10^5$ s$^{-1}$, from the initial value of $2.9 \times 10^5$ s$^{-1}$ for the pure enzyme (Table 1). It may be observed that D-Phe enhances two times more efficiently the $k_{cat}$ of this isozyme as compared to its enantiomer, being thus a better CA VA activator. This phenomenon is slightly more accentuated for hCA VB, case in which the $k_{cat}$ has been augmented from the initial value of $9.5 \times 10^5$ s$^{-1}$ for the pure enzyme to values of 17.6–$33.9 \times 10^5$ s$^{-1}$ in the presence of L-/D-Phe (Table 1). It may be thus observed that D-Phe is a particularly efficient hCA VB activator, increasing the enzyme velocity by a factor of 3.56 (at a concentration of 10 μM of activator), whereas its enantiomer, L-Phe, is a much weaker hCA VB activator (it increases the velocity by a factor of 1.85, at the same concentration mentioned above for D-Phe). Detailed kinetic measurements (i.e., for determining $k_{cat}$ and $K_M$ values) have been done with all the investigated derivatives **1**–**17**, but only values for L-/D-Phe are reported in Table 1, for the sake of simplicity, as the other derivatives behaved similarly with these two CAAs.

hCA VA/hCA VB activation constants ($K_A$s) for a series of structurally related amino acids and amines of types **1**–**17** are shown in Table 2. The activation constants for the ubiquitous isozymes hCA I and hCA II are also provided for comparison. Similarly with the inhibition constant $K_I$ (for the enzyme inhibitors),[1–4] the activation constant $K_A$ measures the affinity of the activator for the enzyme. The lower this parameter is, stronger is the

**Table 1.** Kinetic parameters for the activation of hCA isozymes I, II, VA and VB with L- and D-Phe, at 25 °C and pH 7.5, for the $CO_2$ hydration reaction

| Isozyme | $k_{cat}$[a] ($s^{-1}$) | $(k_{cat})_{L\text{-Phe}}$[b] ($s^{-1}$) | $(k_{cat})_{D\text{-Phe}}$[b] ($s^{-1}$) | $K_A$[c] (μM) | |
|---|---|---|---|---|---|
| | | | | L-Phe | D-Phe |
| hCA I[d] | $2.0 \times 10^5$ | $19.8 \times 10^5$ | $2.3 \times 10^5$ | 0.07 | 86 |
| hCA II[d] | $1.4 \times 10^6$ | $5.7 \times 10^6$ | $5.2 \times 10^6$ | 0.013 | 0.035 |
| hCA VA[e] | $2.9 \times 10^5$ | $4.3 \times 10^5$ | $9.7 \times 10^5$ | 9.81 | 4.63 |
| hCA VB[e] | $9.5 \times 10^5$ | $17.6 \times 10^5$ | $33.9 \times 10^5$ | 10.45 | 0.072 |

[a] Observed catalytic rate without activator. $K_M$ values in the presence and the absence of activators were the same for the various CA isozymes (data not shown).
[b] Observed catalytic rate in the presence of 10 μM activator.
[c] The activation constant ($K_A$) for each isozyme was obtained as described earlier,[20] and represents the mean from at least three determinations by a stopped-flow, $CO_2$ hydrase assay method.[20] Standard errors were in the range of 5–10% of the reported values.
[d] Human recombinant isozymes.
[e] Human recombinant full length isozyme.

**Table 2.** Activation constants of hCA I/hCA II (cytosolic isozymes) and hCA VA/hCA VB (mitochondrial isoforms) with amino acids and amines **1**–**17**

| No. | Compound | $K_A$[a] (μM) | | | |
|---|---|---|---|---|---|
| | | hCA I[b] | hCA II[b] | hCA VA[c] | hCA VB[c] |
| **1** | L-His | 0.03 | 10.9 | 1.34 | 0.97 |
| **2** | D-His | 0.09 | 43 | 0.12 | 4.38 |
| **3** | L-Phe | 0.07 | 0.013 | 9.81 | 10.45 |
| **4** | D-Phe | 86 | 0.035 | 4.63 | 0.072 |
| **5** | L-DOPA | 3.1 | 11.4 | 0.036 | 0.063 |
| **6** | D-DOPA | 4.9 | 7.8 | 4.59 | 3.71 |
| **7** | L-Trp | 44 | 27 | 1.13 | 0.89 |
| **8** | D-Trp | 41 | 12 | 1.24 | 1.35 |
| **9** | L-Tyr | 0.02 | 0.011 | 2.45 | 0.044 |
| **10** | 4-$H_2N$–L-Phe | 0.24 | 0.15 | 2.76 | 2.17 |
| **11** | Histamine | 2.1 | 125 | 0.010 | 3.52 |
| **12** | Dopamine | 13.5 | 9.2 | 0.13 | 7.85 |
| **13** | Serotonin | 45 | 50 | 6.33 | 0.11 |
| **14** | 2-Pyridyl-methylamine | 26 | 34 | 23.56 | 0.24 |
| **15** | 2-(2-Aminoethyl)pyridine | 13 | 15 | 7.62 | 0.094 |
| **16** | 1-(2-Aminoethyl)-piperazine | 7.4 | 2.3 | 6.04 | 0.91 |
| **17** | 4-(2-Aminoethyl)-morpholine | 0.14 | 0.19 | 0.089 | 1.15 |

Data for hCA I and II activation with these compounds (except for **9** and **10**) are from Ref. 15.
[a] Mean from three determinations by a stopped-flow, $CO_2$ hydrase method.[20] Standard errors were in the range of 5–10% of the reported values.
[b] Human recombinant isozymes, stopped-flow $CO_2$ hydrase assay method.[20]
[c] Full length, human recombinant enzyme, stopped-flow $CO_2$ hydrase assay method.[20]

activator against the corresponding isoform.[7,13–15] Compounds **1**–**17** were shown earlier to act as activators of the cytosolic isozymes hCA I, II, and XIII.[7,13–15] All of them possess protonatable moieties of the primary amine or heterocyclic amine type (or both of them), being thus able to participate in proton transfer processes leading to the generation of the nucleophilic species of the enzyme, with the hydroxide anion coordinated to the active site zinc ion. It should be noted that the amines included in our study possess aminoethyl or aminomethyl moieties, in addition to aromatic/heterocyclic groups, the last of which usually incorporate nitrogen atoms that can be protonated at pH values in the physiological range.

Data of Table 2 show that amines and amino acids studied here, of type **1**–**17**, act as CAAs against all four CA isozymes, but with quite different activity profiles for the newly investigated mitochondrial isoforms (hCA VA/hCA VB), as compared to the cytosolic ones (hCAI/hCA II). Thus, the following SAR can be observed from data reported in Table 2: (i) weak CA VA activating effects have been observed with L-Phe **3**, D-Phe **4**, D-DOPA **6**, serotonin **13**, and the structurally related amines **14**–**16**. All these compounds showed activation constants in the range of 4.59–23.56 μM. Medium potency hCA VA activating effects were then observed for the following derivatives: L-His **1**, L-Trp **7**, D-Trp **8**, L-Tyr **9**, and 4-amino-L-Phe **10**, which possessed activation constants in the range of 1.13–2.76 μM. The best hCA VA activators were D-His **2**, L-DOPA **5**, histamine **11**, dopamine **12**, and 4-(2-aminoethyl)-morpholine **17**, which showed $K_A$s in the range of 10–130 nM. The best hCA VA activator identified so far is thus histamine, which with an affinity in the low nanomolar range ($K_A$ of 10 nM) activates very efficiently hCA VA as compared to other investigated isoforms, such as CA I and II (or CA VB, see discussion later in the text), for which

it showed an affinity in the micromolar range. It should be also mentioned that even if the affinity of histamine for CA II is quite low ($K_A$ of 125 μM), the X-ray crystal structure of the hCA II—histamine adduct was the first CA—activator complex for which the tridimensional organization was known at a molecular level,[9] allowing thus a better understanding of the CA activation mechanism and a more rational drug design of different types of CAAs.[21] (ii) Weak hCA VB activating properties have been observed for D-His **2**, L-Phe **3**, D-DOPA **6**, histamine **11**, and dopamine **12**, which showed activation constants in the range of 3.52–10.45 μM (Table 2). Better activators were on the other hand D-Trp **8**, 4-amino-L-Phe **10**, and 4-(2-aminoethyl)-morpholine **17**, which showed $K_A$s in the range of 1.15–2.17 μM. A rather large group of derivatives, such as L-His **1**, D-Phe **4**, L-DOPA **5**, L-Trp **7**, L-Tyr **9**, and the amines **13**–**16**, showed excellent hCA VB activating effects, with $K_A$s in the range of 44–910 nM. The best hCA VB activators were three amino acid derivatives, D-Phe, L-DOPA, and L-Tyr, which with $K_A$s in the range of 44–72 nM effectively activate this isoform. (iii) Important differences of activation power against both hCA VA and hCA VB (but also against the cytosolic isoforms hCA I and II investigated earlier)[7,15] were observed for pairs of enantiomers investigated here (derivatives **1**–**8**). In fact, it has been documented by means of X-ray crystallography that such enantiomers (such as for example L- and D-His,[10,13a] or L- and D-Phe[13b]) bind in a different manner within the enzyme active site, interacting with diverse amino acid residues and participating in the proton transfer processes by means of different pathways. It may be also noted that the stereochemistry of the amino acid pair of compounds was not the crucial element in the CA VA/CA VB activating efficacy, since in some cases the L-enantiomer was a better activator over the corresponding D-enantiomer (L-DOPA and L-Trp against both CA VA and CA VB), whereas in other cases the D-enantiomer was a better activator as compared to the corresponding L-one (D-His and D-Phe against hCA VA, and D-Phe against hCA VB). There was also not a notable difference of activity between amino acids and structurally related amines (compare L-His, D-His, and histamine, or L-DOPA, D-DOPA, and dopamine, respectively), as both types of derivatives showed good hCA VA/B activating properties, but with a great difference of efficiency. Obviously, as this is the first detailed CA VA/CA VB activation study reported up to now, the number of investigated compounds is rather limited for allowing a detailed SAR discussion (and the X-ray crystal structures of hCA VA and hCA VB are not known for the moment). (iv) hCA VB seemed to generally possess a higher affinity for this type of CAAs, with a few notable exceptions, such as for example histamine, which is a 352 times more effective hCA VA than hCA VB activator, and dopamine, a 60 times better hCA VA than hCA VB activator. For example the difference of activity of amine **14** against the four investigated isoforms is quite impressive. This compound is in fact a quite ineffective CA I, II, and VA activator (activation constants in the range of 23.56–34 μM), but is a submicromolar hCA VB activator ($K_A$ of 0.24 μM). Thus, the difference of activation power between hCA VA and hCA VB by this compound is around 100-fold (the precise value is 98.16) (Table 2).

Up to now there are no physiological studies regarding the mitochondrial CA activation (in fact the only such studies regarding the brain CA activation are reports of Sun and Alkon,[11,12] who took into consideration the cytosolic isoforms CA I and II abundant in this organ. However, the brain contains other isozymes too, amongst which CA VB[2]). Considering the excellent activating properties evidenced here for the first time for a panel of biogenic amines and amino acids highly abundant in many tissues/organs in the human body (such as histamine, dopamine, L-His, and L-Phe among others),[22,23] we estimate that our findings may have physiologic relevance. For example, it has been reported that the blood and brain concentrations of L-Phe are quite variable, in the range of 30–73 μM,[22] whereas the concentrations of L-His present in many tissues, including the brain, are in the range of 60–120 mM.[23] These are concentrations at which important hCA VA and hCA VB activating effects should be observed, considering the activation constants reported by us here. Additional studies are warranted for understanding the physiologic/pathologic significance of our findings.

In conclusion, the first detailed hCA VA/hCA VB activation study is reported here. D-His, L-DOPA, histamine, dopamine, and 4-(2-aminoethyl)morpholine were excellent hCA VA activators, with $K_A$s in the range of 10–130 nM. Good hCA VB activating effects were observed for L-His, D-Phe, D-DOPA, L-Trp, L-Tyr, serotonin, and 2-(2-aminoethyl)-pyridine, with $K_A$s in the range of 44–110 nM. All these activators enhanced $k_{cat}$, having no effect on $K_M$, favoring thus the rate-determining step in the catalytic cycle, the proton transfer reactions between the active site and environment. The activation pattern of the two mitochondrial isoforms is very different between each other and as compared to those of the cytosolic isoforms hCA I and II.

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20. Khalifah, R. G. *J. Biol. Chem.* **1971**, *246*, 2561 An Applied Photophysics stopped-flow instrument has been used for assaying the CA-catalyzed $CO_2$ hydration activity. Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 10 mM Hepes (pH 7.5) as buffer, 0.1 M $Na_2SO_4$ (for maintaining constant the ionic strength), following the CA-catalyzed $CO_2$ hydration reaction for a period of 10 s. The $CO_2$ concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters and activation constants. For each activator at least six traces of the initial 5–10% of the reaction have been used for determining the initial velocity. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of activators **1**–**17** (10 mM) were prepared in distilled-deionized water and dilutions up to 0.001 μM were done thereafter with distilled-deionized water. Activator and enzyme solutions were preincubated together for 15 min at room temperature prior to assay, in order to allow for the formation of the E–A complex. The activation constant ($K_A$), defined similarly with the inhibition constant $K_I$, [3,7] may be obtained by considering the classical Michaelis–Menten equation (Eq. 1), which has been fitted by non-linear least squares by using PRISM 3:

$$v = v_{max}/\{1 + K_M/[S](1 + [A]_f/K_A)\} \quad (1)$$

where $[A]_f$ is the free concentration of activator.
Working at substrate concentrations considerably lower than $K_M$ ($[S] \ll K_M$), and considering that $[A]_f$ can be represented in the form of the total concentration of the enzyme ($[E]_t$) and activator ($[A]_t$), the obtained competitive steady-state equation for determining the activation constant is given by Eq. 2:[7]

$$v = v_0 \cdot K_A/\{K_A + ([A]_t - 0.5\{([A]_t + [E]_t + K_A) - ([A]_t + [E]_t + K_A)^2 - 4[A]_t \cdot [E]_t)^{1/2}\}\} \quad (2)$$

where $v_0$ represents the initial velocity of the enzyme-catalyzed reaction in the absence of activator.[7]
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