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Carbonic anhydrase activators. Activation of the membrane-associated isoform XV with amino acids and amines

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ABSTRACT

An activation study of the membrane-associated carbonic anhydrase (CA, EC 4.2.1.1) isoform XV with a series of natural and non-natural amino acids and aromatic/heterocyclic amines is reported. Murine CA XV was strongly activated by some amino acids (D-Phe, L-/D-DOPA, D-Trp, L-Tyr) and amines (dopamine, serotonin, L-adrenaline and 4-(2-aminoethyl)-morpholine) with activation constants in the range of 4.0–9.5 μM. L-/D-His, L-Phe, histamine and several other heterocyclic amines showed less efficient activation (K_As in the range of 11.6–33.4 μM). The activation profile of CA XV is quite different from that of the cytosolic isoforms CA I and II or the membrane-associated CA IV. All mammalian isoforms CA I–XV are thus characterized for their interaction with this set of amino acid and amine activators, some of which are biogenic amines or neurotransmitters present in sufficiently high amounts in various tissues for exerting significant biologic responses.

The activation of various isoforms of the zinc enzyme carbonic anhydrase (CA, EC 4.2.1.1) has been investigated in some detail in the last period.[1–4] Indeed, in mammals 16 such isoforms have been described, possessing various tissue distributions and physiological roles: the cytosolic CA I, II, III, VII and XIII, the mitochondrial CA VA and CA VB, the secreted CA VI (in saliva and milk), the membrane-associated ones CA IV and XV, the transmembrane ones CA IX, XII and XIV, as well as the acatalytic ones CA VIII, X and XI.[5–9] Inhibitors of CAs are in clinical use for more than 50 years, as diuretics, antiglaucoma, antiobesity, anticonvulsants or antitumor drugs/diagnostic tools,[10] whereas activators are not only less investigated but their clinical applications were only marginally explored as potential anti-Alzheimer's disease agents, although several CA isoforms are present in lower amounts in the brain of such patients compared to normal subjects.[11] Furthermore, a deficiency syndrome of the major CA isoform (CA II) has been described and investigated in detail by Sly's group.[12] Patients affected by this genetic disease show normal levels of other isoforms such as for example the catalytically less efficient CA I, whereas their CA II is unstable due to a mutation which renders the protein prone to proteolytic degradation.[13] Thus, activation of CA isoforms (in patients suffering of the CA II deficiency syndrome or those affected by Alzheimer's disease) by specific activators may constitute a new pharmacological approach for the management of these diseases for which few therapeutic options are available to date.[10,11]

By means of detailed kinetic and X-ray crystallographic data[1–4,14] it has been demonstrated that CA activators bind within the enzyme active site, in a region different of the substrate or inhibitor binding sites, and participate in this enzyme–activator complex to the rate determining step of the catalytic cycle, that is, a proton transfer from the zinc bound water to the environment, with generation of the catalytically active enzyme species, possessing a hydroxide ion coordinated to Zn(II).[1–4,10,13,14] As a consequence, most CA activators (CAAs) belong to classes of compounds capable of shuttling protons in the pH range of 6–8, such as amines, amino acid and oligopeptides.[13–15] Mammalian isoforms CA I–CA XIV were investigated for their interaction with these activator classes, whereas the latest such isozyme, CA XV, discovered recently by Parkkila's group[16] and investigated for its catalytic[17] and inhibition properties (with sulfonamides, phenols and inorganic anions) by this group[18–20] has not yet been investigated at all for its activation.

Here we report the first CA XV activation study, with a series of amino acids and amines which were studied earlier for their interaction with the other mammalian isoforms, CA I–XIV.[1–4] Thus, these derivatives, of types **1–18**, have been now assayed for their complete activation profile against all catalytically active mammalian CAs.[21]

The affinity constant (K_{aff}) of an activator for the corresponding CA isoform has been denominated the activation constant (K_A)[1–3,21] in order to obtain a measure of the strength for the interaction

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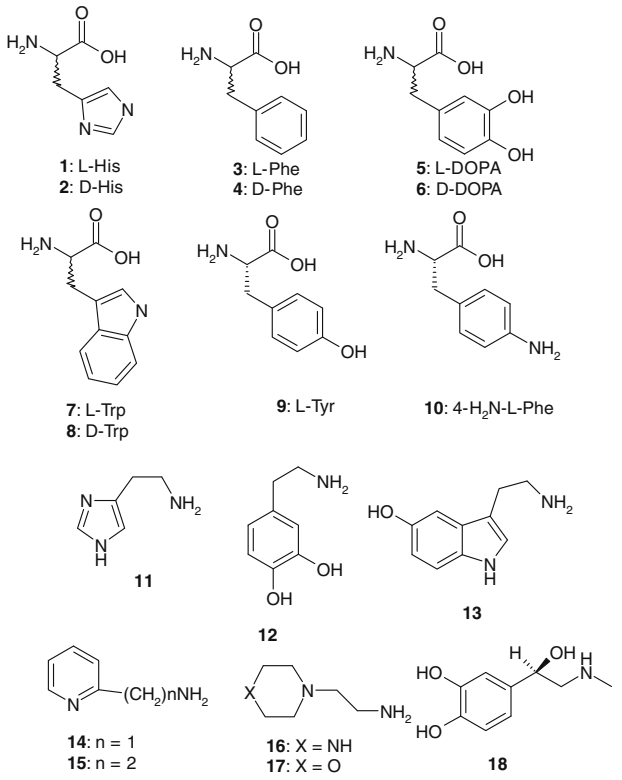

between enzyme and activator, similarly with the inhibition constant (K_I) which defines the potency of an inhibitor in the enzyme–inhibitor (E–I) complex.[1–3] By representing the catalytic enhancement as a function of activator concentration, a typical sigmoid curve is obtained, from which the affinity constant (K_A) may be estimated by non-linear least-squares fitting.[21] Detailed kinetic measurements (Table 1) showed that the activators **1–18** investigated here for their interactions with isoform hCA I, II and IV as well as mCA XV, do not change the value of the Michaelis–Menten constant (K_M), which is the same in the absence or the presence of activators, similarly to that observed earlier for the activation of other mammalian CAs.[1–4,14,15] On the contrary, the observed catalytic rate of the enzyme (k_{cat}) is enhanced in the presence of all activators investigated up to now, and for all CA isozymes (Table 1), supporting our previous observations[1–4,14,15] that CA activators (CAAs) do not influence the binding of CO_2 to the CA active site, but intervene in the rate-determining step of the catalysis, i.e., the transfer of protons from the active site to the environment.

In order to explore structure-activity relationship (SAR) for the activation of mCA XV with compounds **1–18**, the activation constants of these compounds against this new isoform as well as the highly investigated cytosolic isoforms hCA I and II as well as hCA IV (an isoform possessing the highest sequence homology with mCA XV) are presented in Table 2. It should be mentioned that primates do not express CA XV as the gene encoding this protein has been transformed to a pseudogene during evolution.[16] However, most mammals (except primates) and fish species investigated so far do possess active CA XV.[16,17] Data of Table 2 show that all amino acid and amines **1–18** investigated here act as efficient activators against mCA XV. The following SAR can be noted:

(i) several amino acids, such as D-Phe, L-and D-DOPA, D-Trp, and L-Tyr, as well as most amines investigated here (of types **12–18**) effectively activated mCA XV, with activation constants in the range of 4.0–11.9 μM. The best mCA XV activator was D-DOPA **6** (K_A of 4.0 μM) but actually all these derivatives showed a compact behavior of effective CAAs, with little variation of the activation constant (threefold, between 4 and 12 μM). On the contrary, some of these CAAs show a much wider range of activities against the cytosolic isozymes hCA I and II or the membrane-associated one hCA IV. For example, the activation constants of these compounds against hCA I are in the range of 20 nM (L-Tyr)–86 μM (L-Phe), whereas for the activation of hCA II this range is even wider: 11 nM (L-Tyr)–125 μM (histamine). It is difficult to explain these data without an X-ray crystal structure of mCA XV and with the relatively few hCA I/II–activator adducts characterized so far by means of this technique.[11,13,14]Among the amines **12–18** acting as efficient mCA XV activators, it may be observed that both aromatic

Table 1
Activation of hCA isozymes I, II, IV and mCA XV, with L- and D-histidine, at 25 °C, for the CO_2 hydration reaction

Isozyme	k_{cat}[a] (s^{-1})	$(k_{cat})_{L\text{-}His}$[b] (s^{-1})	$(k_{cat})_{D\text{-}His}$[b] (s^{-1})	K_A[c] (μM)	
				L-His	D-His
hCA I[d]	2.0×10^5	13.4×10^5	9.1×10^5	0.03	0.09
hCA II[d]	1.4×10^6	4.3×10^6	2.7×10^6	10.9	43.5
hCA IV[e]	1.2×10^6	4.3×10^6	3.8×10^6	7.3	12.3
mCA × V[f]	4.7×10^5	8.5×10^5	15.0×10^5	32.1	14.1

[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 (i.e., 4.0 mM for hCA I; 9.3 mM for hCA II; 21.5 mM for hCA IV and 14.2 mM for mCA XV, respectively).
[b] Observed catalytic rate in the presence of 10 μM activator.
[c] The activation constant (K_A) for each isozyme was obtained by fitting the observed catalytic enhancements as a function of the activator concentration.[21] Mean from at least three determinations by a stopped-flow, CO_2 hydrase method.[21] Standard errors were in the range of 5–10% of the reported values.
[d] Human recombinant isozymes.
[e] Truncated human recombinant isozyme lacking the first 20 amino acid residues which represent the signal peptide orienting the protein outside the cell.[14]
[f] Murine recombinant isoform.[20b]

Table 2
Activation constants of hCA I, hCA II (cytosolic isoforms), and hCA IV and mCA XV (membrane-associated isoform), with amino acids and amines **1–18**

No.	Compound	K_A (μM)[a]			
		hCA I[b]	hCA II[b]	hCA IV[c]	mCA XV[d]
1	L-His	0.03	10.9	7.30	32.1
2	D-His	0.09	43	12.3	14.1
3	L-Phe	0.07	0.013	36.3	33.4
4	D-Phe	86	0.035	49.3	9.5
5	L-DOPA	3.1	11.4	15.3	6.5
6	D-DOPA	4.9	7.8	34.7	4.0
7	L-Trp	44	27	37.1	13.5
8	D-Trp	41	12	39.6	8.7
9	L-Tyr	0.02	0.011	25.1	8.9
10	4-H_2N-L-Phe	0.24	0.15	0.079	16.3
11	Histamine	2.1	125	25.3	18.5
12	Dopamine	13.5	9.2	30.9	7.1
13	Serotonin	45	50	3.14	7.5
14	2-Pyridyl-methylamine	26	34	5.19	11.6
15	2-(2-Aminoethyl)pyridine	13	15	7.13	11.9
16	1-(2-Aminoethyl)-piperazine	7.4	2.3	24.9	10.4
17	4-(2-Aminoethyl)-morpholine	0.14	0.19	1.30	9.3
18	L-Adrenaline	0.09	96	45.0	6.9

Activation data of hCA I, II and IV with these compounds are from Ref. 15a.
[a] Mean from three determinations by a stopped-flow, CO_2 hydrase method.[21] Standard errors were in the range of 5–10% of the reported values.
[b] Human recombinant isozymes, stopped flow CO_2 hydrase assay method.[21]
[c] Human recombinant enzyme lacking the first 20 aminoterminal residues,[14] stopped flow CO_2 hydrase assay method.[21]
[d] Murine recombinant isoform.[20b]

(dopamine **12**, L-adrenaline **18**) as well as heterocyclic derivatives (**13–17**) incorporating various ring systems, possess these properties, again, with small variations in the range of the activation constants (between 7.1–11.9 M, Table 2).

(ii) The remaining compounds, that is, L- and D-His, L-Phe, L-Trp, 4-amino-phenylalanine and histamine, showed slightly less effective mCA XV activating properties compared to the compounds discussed above, with K_As in the range of 13.5–33.4 μM. It may be observed that small structural changes in the activator molecule strongly influence the interaction with the enzyme(s). For example, the two enantiomers of the same amino acid showed quite distinct CA activating effects. In this case, against mCA XV, all D-amino acids were more effective CAAs compared to the corresponding L-enantiomer, sometimes by a factor of 3.5 (D- vs L-Phe), whereas for isoforms hCA I, II and IV, generally just the opposite was true,[13,14] with the L-enantiomers more effective as activators compared to their D-counterparts (Table 2). The presence of an additional functional group, such as the OH or H_2N on the Phe scaffold, also leads to important differences of activity, with L-DOPA and L-Tyr being more effective mCA XV activators compared to the parent molecule L-Phe, by a factor of 5.1 and 3.7, respectively. Such effects were in fact also observed for the interaction of some of these compounds with other CA isozymes, and explained by means of detailed X-ray crystallographic work of CA–activator adducts.[11,13,14] These studies demonstrated that the two enantiomers of the same amino acid (e.g., L- and D-His; L-and D-Phe)[14,15] bind in very different modes to the enzyme active site (CA II) and also that the same activator (L-His) binds with different orientations and conformations within the active site of two diverse isoforms, such as CA I and II.[22]

(iii) The activation profile of mCA XV with these compounds is quite distinct from those of the cytosolic isoforms hCA I and II or the membrane-bound one hCA IV (Table 2). This is not surprising after all, as we have shown that the different 13 CAs of mammalian origin are all activated by these compounds, but with profiles which are typical for each particular isoform.[1–3,14,15]

In order to rationalize this behavior, we shall compare the sequence of mCA XV with that of hCA IV, since this is the enzyme with which CA XV shares the highest sequence similarity.[16] Furthermore, similarly to CA IV, CA XV is tethered to the plasma membrane by means of a glycosylphosphatidylinositol tails,[16,17] and the active site o both isoforms is outside the cell.

Data of Figure 1 show that among hCA IV and mCA XV, many amino acid residues are conserved between these two isozymes, including important residues involved in the catalytic cycle: (i) the three zinc ligands, His104, 106, and 129 (mCA XV numbering system);[20b] (ii) the 'gate-keeping' residues Thr213 and Glu116, which orient the substrate in the right position to be attacked by the zinc-bound hydroxide ion; and (iii) His72, the proton shuttle residue, which transfers protons from the zinc bound water molecule towards the external medium, leading to the generation of the active form of the enzyme with hydroxide as the fourth zinc ligand.[1–4] It may be observed that many other amino acids are common between the two isoforms, but most of them are outside the active site (Fig. 1). However, some of the amino acid residues involved in the binding of activators (as shown by X-ray crystallography of CA–activator adducts)[1–4,13,14] are different in mCA XV compared to the other isoforms. Thus, the following amino acids were shown to be involved in the binding of amine and amino acid activators (hCA I numbering system): His64, Asn67, Gln92, and Thr200.[13,14] These amino acids correspond to Leu67, His72, Gln102 and Thr214 in the mCA XV sequence (Fig. 1, mCA XV numbering system). Obviously His72 is involved in the catalytic cycle (as the proton shuttle residue), whereas Thr214 is very near the gate-keeper residue Thr213, and it would be difficult to change these residues without compromising the catalytic efficiency of the isozyme. Thus, it is not unexpected that they are also conserved in the mCA XV sequence. However, the replacement of the rather hydrophilic Asn67 side chain (present in most cytosolic CA isozymes, such as CA I and II,[1–3,20b] but which is not at all conserved in the membrane-associated ones, being an Ala in hCA IV and a Leu in mCA XV) by the more lipophilic Leu chain in mCA XV, may lead to the different binding of activators reported here. We do not want to imply that just one amino acid is responsible for the activation profile of mCA XV reported here, but we hypothesize that this hydrophobic side chain of Leu67 may be one of the important structural elements responsible for this particular activation profile, typical of this isoform. Obviously, the overall shape and polarity of this enzyme's active site are as important as the particular residues involved in the binding of a modulator of activity, as showed by extensive crystallographic work on other CA isozymes. Work is in progress in these laboratories for verifying these hypotheses.

In conclusion, we investigated the interaction of the membrane-associated isoform CA XV with a series of natural and non-natural amino acids and aromatic/heterocyclic amines. CA XV was strongly activated by some amino acids (D-Phe, L-/D-DOPA, D-Trp, L-Tyr) and amines (dopamine, serotonin, L-adrenaline and 4-(2-aminoethyl)-morpholine) with activation constants in the range of 4.0–9.5 μM. L-/D-His, L-Phe, histamine and several other heterocyclic

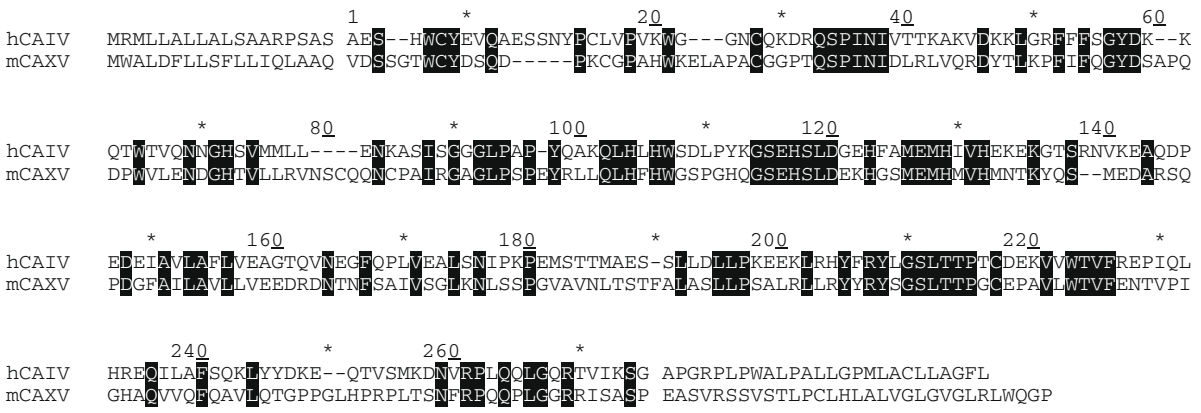

Figure 1. Alignment of hCA IV and mCA XV. The numbering is according to mature mCA XV, and the alignment was made as described previously.[20b] The positions shaded in black have identical residues in both isozymes. The histidine residues that bind the Zn(II) ion and are crucial for the CA catalytic activity are represented by the ■ symbol.

amines showed less efficient activation (K_As in the range of 11.6–33.4 μM). The activation profile of CA XV is quite different from that of the cytosolic isoforms CA I and II or the membrane-associated one CA IV, which has the highest identity of the amino acid sequence with CA XV. All mammalian isoforms CA I–XV have been now characterized for their interaction with this set of amino acid and amine activators.

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21. Khalifah, R. G. *J. Biol. Chem.* **1971**, *246*, 2561.An applied photophysics stopped-flow instrument was used for assaying the CA catalyzed CO_2 hydration activity. Phenol red (at a concentration of 0.2 mM) was 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 ionic strength), following the CA-catalyzed CO_2 hydration reaction for a period of 10 s at 25 °C. 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**–**18** (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,[1–3] can be obtained by considering the classical Michaelis–Menten equation (Eq. 1), which has been fitted by non-linear least squeares 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:[14,15]

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

where v_0 represents the initial velocity of the enzyme-catalyzed reaction in the absence of activator.[14,15]
22. Temperini, C.; Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 5152.