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Carbonic anhydrase activators. The first activation study of a coral secretory isoform with amino acids and amines

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ABSTRACT

The activity of the coral *Stylophora pystillata* secretory carbonic anhydrase STPCA has been tested in presence of amino acids and amines. All the investigated compounds showed a positive, activating effect on k_{cat} and have been separated in weak (K_A in the range of 21–126 μM), medium (10.1–19 μM) and strong enzyme activators (K_A of 0.18–3.21 μM). D-DOPA was found to be the best coral enzyme activator, with an activation constant K_A of 0.18 μM. This enhancement of STPCA activity, as well as previous enzyme inhibition results, might now be tested on living organisms to better understand the role played by these enzymes in the coral calcification processes.

1. Introduction

Carbonic anhydrases (CA, EC 4.2.1.1) are ubiquitous metalloenzymes that catalyze the reversible hydration of carbon dioxide into bicarbonate and protons: $CO_2 + H_2O \Longleftrightarrow HCO_3^- + H^+$. So far, there are at least five classes of CAs with polyphyletic origin and named α-, β-, γ-, δ- and ξ-CA.[1–6] These enzymes are involved in many physiological processes such as pH regulation, respiration, secretion of electrolytes, biosynthesis of some important biomolecules such as urea, glucose, lipids and pyrimidines, excretion of acid and salts, carcinogenesis, and signaling.[7–13] Among all of these roles, the CAs are involved in biomineralization from invertebrates to vertebrates, although the ensuing mechanisms are poorly understood at this moment.[14–18]

Scleractinian corals are major biomineralizing organisms which precipitate an aragonitic calcium carbonate ($CaCO_3$) skeleton. The first evidence of the involvement of CA in coral calcification came from Goreau in 1959.[14] Indeed, he demonstrated that the sulfonamide CA inhibitor acetazolamide reduces calcification rates. Numerous subsequent studies have then confirmed that sulfonamides (acetazolamide or ethoxzolamide) inhibit calcification rates up to 73% being thus suggested that CAs are involved in the inorganic carbon supply for calcification and/or the regulation of pH at the calcification site.[19–22] We have recently cloned, sequenced and localized an α-CA from the coral *Stylophora pistillata*, named STPCA.[23] STPCA is a secreted isoform and, owing to its specific secretion by the calicoblastic calcifying ectoderm, we proposed that this enzyme plays a direct role in biomineralization. Furthermore, we performed the first inhibition studies, by simple anions or sulfonamides/sulfamates, of an invertebrate CA at the molecular level.[24,25] From these studies it appears that STPCA has a catalytic activity for the CO_2 hydration reaction similar to that of the extracellular secreted hCA VI. Indeed, STPCA is less effective as a catalyst for the CO_2 hydration reaction, as compared to the very rapid isozymes hCA II (cytosolic) or hCA IX (transmembrane).[1,26,27]

Owing to the wide range of function that CAs play, they became commonly studied drug targets. Inhibitors of these enzymes show many pharmacological applications such as management of glaucoma, diuretics, anticonvulsants, antiobesity and/or antitumor agent/diagnostic tools.[28] Activators started to be investigated more recently.[29] Previous[13,29–34] data on the activation CAs clearly showed that a CA activator (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, but should interact favorably with amino acid residues present in the activator binding pocket, and second, it should possess a moiety able to participate in proton transfer between the active site and the environment, which is the rate-determining step of the catalytic cycle. A multitude of physiologically relevant compounds such as amino acids, oligopeptides or small proteins, as well as many biogenic amines (histamine, serotonin, and catecholamines among others), were shown to efficiently activate the catalytic activity of several CA isozymes, such as CA I, II, IV, IX and XII.[13,29,30,38]

In this work, we present the first activation study of a coral secretory isoform with several amino acids and amines.

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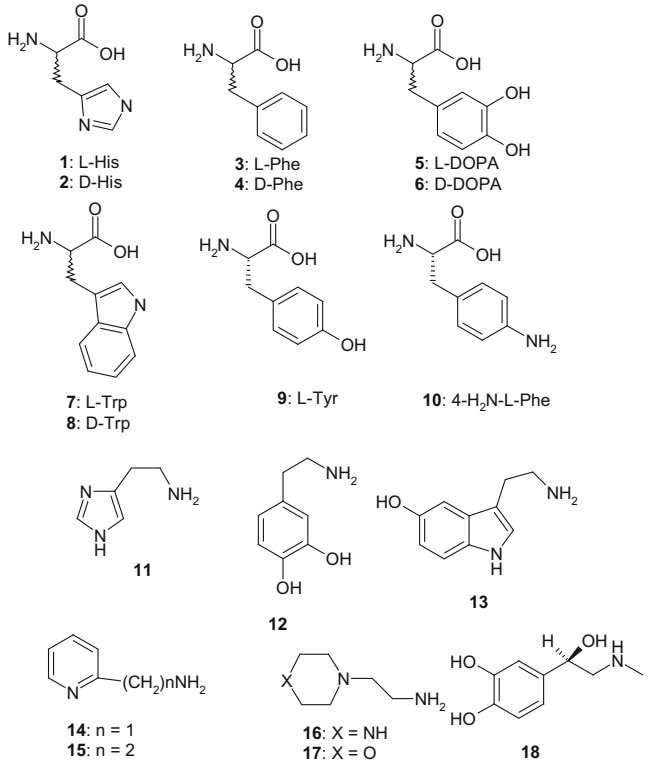
2. Results and discussion

2.1. STPCA kinetic studies

L-/D-Amino acids **1–10** and amines **11–18** were commercially available from Sigma–Aldrich (Milan, Italy) and were used without further purification.



Kinetic experiments for the physiological reaction (carbon dioxide hydration to bicarbonate and a proton) (Table 1) showed that as for hCA I and II,[13,31–33,35] and hCA VI[34] activators of the amino acid or amine type, enhanced k_{cat} of the STPCA enzyme, with no effect on K_M.

As for previously tested human isoforms CA I, CA II and CA VI, 10 μM L- or D-Trp (compounds **7** and **8**) produced a positive effect on the k_{cat} of STPCA without any effect on the K_M value (Table 1). The initial K_{cat} of hCA I ($2.0 \times 10^5\ s^{-1}$) becomes 2.9 and $2.3 \times 10^5\ s^{-1}$ in presence of 10 μM L-Trp and D-Trp, respectively. For hCA II the enhancement of the initial k_{cat} ($1.4 \times 10^6\ s^{-1}$) is in the range of 4.6–6.1 $\times 10^6\ s^{-1}$. For hCA VI the initial k_{cat} ($3.4 \times 10^5\ s^{-1}$) is enhanced in the range of 9.5–4.8 $\times 10^5\ s^{-1}$. The initial STPCA k_{cat} is $3.4 \times 10^5\ s^{-1}$ whereas it is $9.2 \times 10^5\ s^{-1}$ and $6.4 \times 10^5\ s^{-1}$ in presence of L-Trp and D-Trp.

Table 1
Kinetic paramaters for the activation of hCA isozymes I, II, VI and STPCA with L- and D-Trp, at 25 °C and pH 7.5, for the CO_2 hydration reaction

Isozyme	k_{cat}[A] (s^{-1})	$(k_{cat})_{L\text{-}Trp}$[B] (s^{-1})	$(k_{cat})_{D\text{-}Trp}$[B] (s^{-1})	K_A[C] (μM)	
				L-Trp	D-Trp
hCA I[a]	2.0×10^5	2.9×10^5	2.3×10^5	44	41
hCA II[a]	1.4×10^6	4.6×10^6	6.1×10^6	27	12
hCA VI[b]	3.4×10^5	9.5×10^5	4.8×10^5	15	39
STPCA	3.4×10^5	9.2×10^5	6.4×10^5	3.21	19

[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 in Section 4, and represents the mean from at least three determinations by a stopped-flow, CO_2 hydrase method.[19] Standard errors were in the range of 5–10% of the reported values.
[a] Human recombinant isozymes.
[b] Human recombinant full length isozyme.

2.2. Screening activators against STPCA

The activation constants (K_As) of hCA I, hCA II, hCA VI and STPCA presented in Table 2 show that amino acids and amines **1–18** produce different effects on each pure enzyme.

It should be noted that strong activators of cytosolic isoforms hCA I and hCA II, that is, with affinities in the nanomolar range (L-His **1**, D-His **2**, L-Phe **3**, D-Phe **4**, L-Tyr **9**, 4-HN_2-L-Phe **10**, the morpholine derivative **17** and L-adrenaline **18**) are generally weaker activators for the secreted forms hCA VI and STPCA.

All the investigated molecules showed an effect on STPCA activity, which can be separated in different categories, as follows:

(i) Compounds **1–4**, **9**, **11–15**, **17** and **18**, with K_As in the range of 21–126 μM, are weak CASTP activators. Among them, **11–15** are quite ineffective on all tested enzymes (K_As in the range of 9.2–126 μM) with the exception of the relatively good efficiency of Histamine **11** on hCA I and hCA VI.
(ii) With K_As ranging from 10.1 to 19 μM, L-DOPA **5**, D-Trp **8**, 4-H_2N-L-Phe **10** and the piperazine derivative **16** show medium activatory effects.
(iii) The best activators of CASTP are L-Trp **7** and D-DOPA **6** with respective K_As of 3.21 and 0.18 μM. D-DOPA is the only tested compound to have such a high efficiency on STPCA with a K_A in the range of the nanomolar. D-DOPA is also more than eighty times more efficient than its enantiomer L-DOPA **5**. Such a difference between L- and D-enantiomers is also found for the activation of hCA I by L- and D-Phe, L-Phe being ca. 1200 times more efficient than D-Phe. For other compounds, the stereochemistry showed less striking effects, either on STPCA or on human isoforms CA I, CA II and CA VI. These differences probably come from how each enantiomer binds to the active site.

Table 2
Activation constants of hCA I, hCA II, hCA VI and STPCA with amino acids and amines **1–18**

No.	Compound	K_A[A] (μM)			
		hCA I[a]	hCA II[a]	hCA VI[b]	STPCA
1	L-His	0.03	10.9	32	28
2	D-His	0.09	43	13	26
3	L-Phe	0.07	0.013	1.23	34
4	D-Phe	86	0.035	16	21
5	L-DOPA	3.1	11.4	18	15
6	D-DOPA	4.9	7.8	4.58	0.18
7	L-Trp	44	27	15	3.21
8	D-Trp	41	12	39	19
9	L-Tyr	0.02	0.011	9.31	31
10	4-H_2N-L-Phe	0.24	0.15	5.32	10.1
11	Histamine	2.1	125	6.50	120
12	Dopamine	13.5	9.2	21	89
13	Serotonin	45	50	19	56
14	2-Pyridyl-methylamine	26	34	14	126
15	2-(2-Aminoethyl)pyridine	13	15	18	107
16	1-(2-Aminoethyl)-piperazine	7.4	2.3	9.54	11.5
17	4-(2-Aminoethyl)-morpholine	0.14	0.19	42	64
18	L-Adrenaline	0.09	96	58	47

[A] Mean from three determinations by a stopped-flow, CO_2 hydrase method.[19] Standard errors were in the range of 5–10% of the reported values.
[a] Human recombinant isozymes, stopped-flow CO_2 hydrase assay method.[19]
[b] Full length, human recombinant enzyme, stopped-flow CO_2 hydrase assay method.[19]

By mean of X-ray crystallography it is possible to identify with which amino acids does a drug interact and how it plays a part in the proton transfer processes (for a review, see[36]). Indeed, in many mammalian isozymes a His residue (His64, CA I numbering) or a His cluster (His3, 4, 10, 15 and 64, in CA II) serves the proton transfer. The presence of a CAA is then able to increase the number and diversity of proton release pathways, resulting in an enhancement of K_{cat}, that is, a higher rate of catalytic turnover.[37] As example, it has been shown for hCA II that the pattern of hydrogen bonds and hydrophobic contacts with amino acid residues is completely different between L-Phe that forms two hydrogen bonds with Trp5 and His64, and D-Phe that also binds to Thr200 and Pro201 by two other hydrogen bonds. This example illustrates the importance of both the CA activator and the active site structures.[36] Again, like activity[23] and inhibition by anions[24] studies, the present results confirm that STPCA shares more similarities with the secreted hCA VI than with cytosolic hCA I, hCA II or transmembrane hCA IX and hCA XII.[38]

3. Conclusions

Inhibition studies, by acetazolamide and ethoxzolamide, allowed us to show the involvement of CAs in coral calcification. Here, we present the first activation study of a CA in corals. The major part of amino acids and amines tested in this first study of the activation of a coral purified CA were micromolar activators. The best activator was found to be D-DOPA, with a K_A of 0.18 μM. As early as 1948, it has been shown that scleractinian corals calcify faster in the light than in the dark;[39] a phenomenon largely confirmed by Goreau[14] and subsequently called light-enhanced calcification (LEC). However, after more than a half century of research, the mechanism underlying this process remains largely unknown. Among numerous hypotheses,[40–42] Vandermeulen et al.[43] proposed a role of the symbiotic dinoflagellates, from the genus *Symbiodinium*, with which corals can establish a mutualistic phototrophic association. These symbionts would be able to participate to the synthesis of the organic matrix (OM) present in the coral skeleton by providing amino acids and/or precursors of this OM.[44–46] In the light of the present results, we can thus also hypothesize that such compound might play a role in bicarbonate chemistry modulating a CA activity. So far, the nature and structure of these precursors and amino acids are still unknown. In the last decades CAA research was partly relaunched by the evidence that genetic deficiency causes severe physiological and pathological disorders, which might be corrected through an appropriate activation of the enzyme involved.[35,47] At the same time, coral reefs worldwide were facing global warming, especially one of its major effect: ocean acidification. It has been shown that at low pH, the calcification rates of many coral species drop and can even stop.[48] If CA activity is affected, leading to a deficiency in bicarbonate supply for calcification, the use of CAAs might represent a future axis of research in coral physiology and maintenance. Indeed this work, as previous results on sulfonamides/sulfamates inhibition study,[25] must be confirmed by in-vivo assay to better understand the role of carbonic anhydrases and more specifically the role played by STPCA in the calcification process. Moreover, the X-ray crystallographic study of STPCA will be useful to unravel the manner any drug can act on the enzyme.

4. Experimental

4.1. Chemistry

Compounds **1–18** are commercially, highest purity available derivatives from Sigma–Aldrich (Milan, Italy) and were used without further purification.

4.2. CA activation assay

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 inhibition 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 0.1 nM 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 enzyme concentrations in the assay system were: 14 nM for hCA I; 8.3 nM for hCA II; 25.7 nM for hCA VI and 21.3 nM for STPCA, respectively. The activation constant (K_A), defined similarly with the inhibition constant K_I,[9] 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:[13]

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

where v_0 represents the initial velocity of the enzyme-catalyzed reaction in the absence of activator.[13]

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