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Carbonic anhydrase activators: Activation of the human isoforms VII (cytosolic) and XIV (transmembrane) with amino acids and amines

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Abstract—An activation study of the human carbonic anhydrase (hCA, EC 4.2.1.1) isozymes VII and XIV using a small library of natural/non-natural amino acids and aromatic/heterocyclic amines is reported. hCA VII was efficiently activated by L-/D-His, dopamine and serotonin (K_As of 0.71–0.93 μM). The best hCA XIV activators were histamine (K_A of 10 nM), L-Phe, L-/D-His and 4-amino-L-Phe (K_As of 0.24–2.90 μM). In view of the significant expression levels of CA VII and CA XIV in the brain, selective activation of these isoforms may be useful when developing pharmacologic agents for the management of major disorders such as epilepsy and Alzheimer's disease.

Detailed studies on the activation of the zinc enzyme carbonic anhydrase (CA, EC 4.2.1.1) were started after the report ten years ago[1] of the first X-ray crystal structure of an adduct of the ubiquitous human isoform hCA II with histamine. So far, the activation mechanisms of many of the 16 presently known mammalian isoforms have been described at the molecular level.[1–3] It has been shown that CA activators (CAAs)[2,3] intervene within the catalytic cycle of the enzyme in a very specific and interesting manner. Whereas CA inhibitors (CAIs)[3–5] bind to the catalytic Zn(II) ion displacing the fourth zinc ligand, a hydroxide ion/water molecule acting as nucleophile in catalysis, activators bind at the entrance of the active site cavity, in a region in which the proton shuttle residue His64 is placed.[1–3] This action of CAAs facilitates the rate-determining step of the CA catalytic cycle,[1–5] that is, the proton transfer reactions between the zinc-coordinated water molecule and the reaction medium, supplementing thus the proton-shuttling capacity of His64 (or other amino acid residues playing this role in isoforms different from CA II). This leads to a facilitated formation of the nucleophilic (zinc hydroxide) species of the enzyme.[1–5] Indeed, many typical CAAs are able to increase k_{cat} (without having an effect on K_M) for CO_2 hydration to bicarbonate and protons up to 10-fold.[3,6–10] This leads to a drastic enhancement of the catalytic power of these enzymes, some of which are already among the most efficient catalysts that nature has 'designed' (hCA II is one of the fastest enzymes known until now with a k_{cat}/K_M of 1.5×10^8 M^{-1} s^{-1}).[1,3]

Among the 16 CA isoforms characterized so far in mammals, CA I, CA II, CA IV, CA VA, CA VB, CA VI and CA XIII have been investigated in detail with regard to their activation by many classes of compounds, most of which belong to the aromatic/heterocyclic amines, amino acids and small oligopeptide classes.[3,6–12] Furthermore, X-ray crystal structures are available at high resolution for complexes of hCA I and hCA II with activators such as histamine, D-/L-histidine, D-/L-phenylalanine and L-noradrenaline.[1,6–8] This may be helpful for designing isozyme-specific and high-affinity derivatives, some of which may turn out to be useful in the treatment of various kinds

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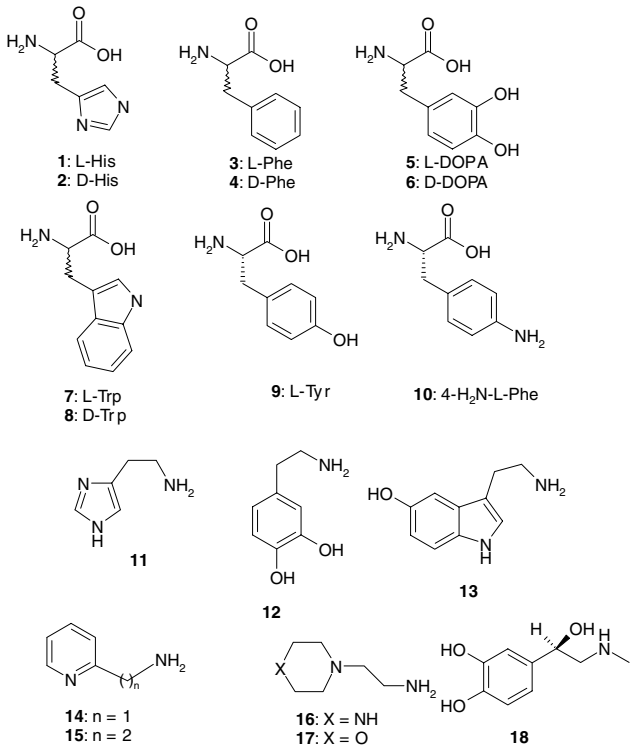
of diseases.[2] However, although the activation of many CA isoforms in vitro is understood in detail,[1,3,6–12] there are very few in vivo studies[13] investigating these phenomena.

No detailed CA activation studies are available of the isoforms CA VII and XIV which show prominent expression in the brain. Notably, CA VII shows a high level of expression in central neurons such as cortical pyramidal cells and cerebellar Purkinje neurons.[14,15] The postnatal up-regulation of intrapyramidal CA VII in rat hippocampus has been shown to closely parallel the generation of high-frequency stimulation (HFS)-induced after-discharges in the hippocampus.[15] Such results point to a crucial role of the developmental expression of CA VII activity in shaping long-term plasticity and promoting epileptogenesis.[15] In addition, its potent inhibition by sulfonamides and sulfamates showed that this isoform is a potential target for the design of anticonvulsant (i.e., 'antiepileptic') drugs.[16] CA XIV has a wide distribution, being present in the brain as well as in the kidney and liver.[17]

In this communication, we present the first detailed study regarding the activation of the two isoforms above, that is, hCA VII (cytosolic) and hCA XIV (a transmembrane isozyme with an extracellular catalytic site). In view of the expression of these isoforms in the brain, the activators studied here include a series of amino acids and amines, some of which are important neurotransmitters or neuromodulators.[15,16]

L-/D-Amino acids and amines **1**–**18** investigated were available from Sigma–Aldrich (Milan, Italy) and were used without further purification. The recombinant CA isozymes (hCA I, II, VII and XIV) have been obtained as described earlier.[16a,18]



Kinetic experiments on the physiologically relevant reaction (carbon dioxide hydration to bicarbonate and a proton) (Table 1)[19] showed that in a manner similar to what was previously observed with hCA I and II,[6–12] activators of the amino acid or amine type enhance k_{cat} of the enzyme, with no effect on K_M. The data in Table 1 show that L-and D-Phe activate all investigated isoforms with regard to CO_2 hydration, but in a very different manner. It should be noted that there are two types of CA isoforms from the catalytic viewpoint: the lower activity ones (CA I-like), including hCA I and hCA XIV (k_{cat} values in the range of $2.0–3.1 \times 10^5\ s^{-1}$), and the high activity ones (CA II-like), among which hCA II and hCA VII (k_{cat} values in the range of $0.95–1.4 \times 10^6\ s^{-1}$, Table 1). It must be stressed that the activators had no influence on the K_M values (data not shown), as the Michaelis–Menten constants were identical with or without activator, but a very strong influence has been observed on k_{cat} (Table 1). This parameter was greatly enhanced in the presence of CAAs, demonstrating that the rate-determining step in catalysis, that is, the proton transfer reactions between the active site and the reaction medium, is facilitated by the activators, as already shown in our first CA activation study with histamine and hCA I/hCA II.[1]

Thus, the activation mechanism of the newly investigated isoforms hCA VII and XIV seems to be identical to that of the better studied isoforms hCA I and II, for which detailed X-ray crystallographic studies of enzyme-activator adducts are available.[1,6–8] This is after all not surprising, since, as shown in Figure 1 where an alignment of the amino acid residues of isoforms I, II, VII and XIV is presented, many active site residues involved in the catalytic cycle (represented by a combination of z, + and * signs) are identical for these 4 isoforms. These include among others: (i) the zinc(II) ion-coordinating residues His94, 96 and 119 (hCA I numbering system); (ii) the proton shuttle (His64); and (iii) the gate-keeping residues Thr199 and Glu106 which orient the substrate molecule (CO_2)

Table 1. Kinetic parametters for the activation of hCA isozymes I, II, VII and XIV with L- and D-Phe, at 25 °C and pH 7.5, for the CO_2 hydration reaction[19]

Isozyme	k_{cat}[c] (s^{-1})	$(k_{cat})_{L\text{-Phe}}$[d] (s^{-1})	$(k_{cat})_{D\text{-Phe}}$[d] (s^{-1})	K_A[e] (μM)	
				L-Phe	D-Phe
hCA I[a]	2.0×10^5	19.8×10^5	2.3×10^5	0.07	86
hCA II[a]	1.4×10^6	5.7×10^6	5.2×10^6	0.013	0.035
hCA VII[b]	9.5×10^5	14.6×10^5	15.8×10^5	10.93	9.74
hCA XIV[a]	3.1×10^5	12.5×10^5	6.1×10^5	0.24	7.21

Standard errors were in the range of 5–10% of the reported values.
[a] Human recombinant isozymes.
[b] Human recombinant full length isozyme.[16a,18]
[c] 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).
[d] Observed catalytic rate in the presence of 10 μM activator.
[e] The activation constant (K_A) for each isozyme was obtained as described earlier[19] and represents the mean from at least three determinations by a stopped-flow, CO_2 hydrase assay method.[19]

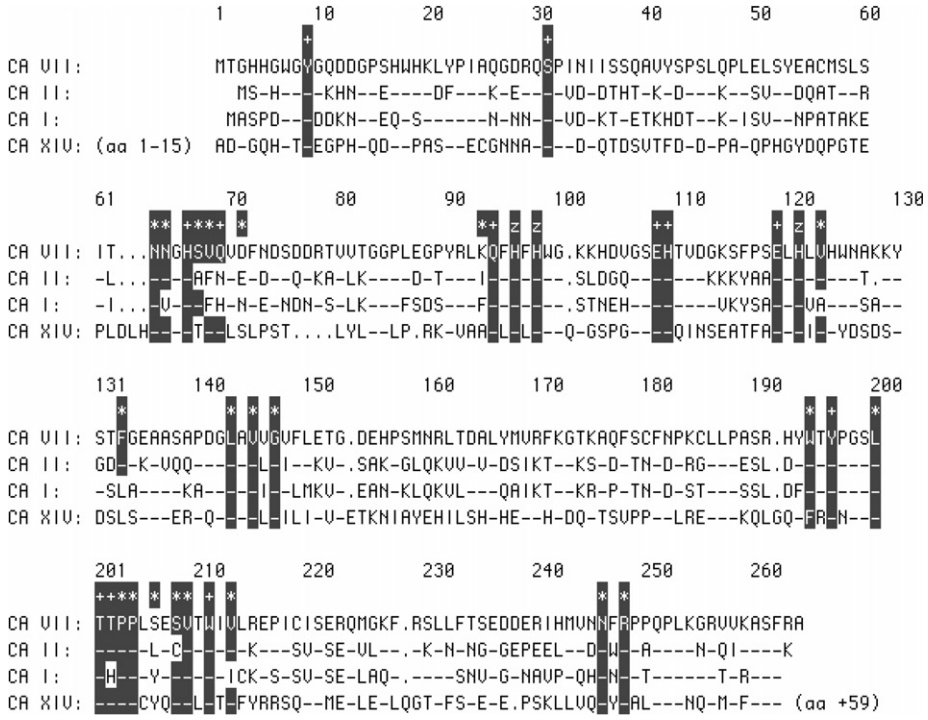


Figure 1. Alignment of the CA VII, I, II (cytosolic isoforms) with the CA XIV (transmembrane isoform) sequence. CA VII is the most archaic[4] cytosolic isoform known and has been used as template for the other sequences. Residues involved in the active site architecture are represented by a combination of z, + and ∗ signs (hCA I numbering). Grey bars represent conserved amino acid residues in all these four isozymes (the '-' sign within or outside the grey bar means that the same amino acid is present as the one shown in the CA VII sequence).

properly for the nucleophilic attack.[20] Obviously, there are also many differences in the active site architecture and amino acid composition of these isoforms, which explain their different catalytic activity, affinity for inhibitors and activators (see below) (Fig. 1).

The data in Table 2 indicate that, as shown for the ubiquitous cytosolic isozymes hCA I and II investigated earlier,[7–12] hCA VII and XIV are also activated by amino acid and amines **1**–**18** in a quite distinct manner. This leads to activation profiles typical for

Table 2. Activation constants of hCA I/hCA II and hCA VII (cytosolic isozymes), as well as hCA XIV (transmembrane isoform) with amino acids and amines **1**–**18**

No.	Compound	K_A (μM)[c]			
		hCA I[a]	hCA II[a]	hCA VII[b]	hCA XIV[b]
1	L-His	0.03	10.9	0.92	0.90
2	D-His	0.09	43	0.71	2.37
3	L-Phe	0.07	0.013	10.93	0.24
4	D-Phe	86	0.035	9.74	7.21
5	L-DOPA	3.1	11.4	58.3	12.1
6	D-DOPA	4.9	7.8	34.7	36.8
7	L-Trp	44	27	57.5	16.5
8	D-Trp	41	12	39.6	18.0
9	L-Tyr	0.02	0.011	20.3	21.8
10	4-H_2N-L-Phe	0.24	0.15	18.7	2.90
11	Histamine	2.1	125	37.5	0.010
12	Dopamine	13.5	9.2	0.89	14.6
13	Serotonin	45	50	0.93	6.5
14	2-Pyridyl-methylamine	26	34	43.7	21.7
15	2-(2-Aminoethyl)pyridine	13	15	27.8	6.9
16	1-(2-Aminoethyl)-piperazine	7.4	2.3	32.5	18.3
17	4-(2-Aminoethyl)-morpholine	0.14	0.19	64.3	5.4
18	L-Adrenaline	0.09	96	60	36.1

Data for hCA I and II activation with these compounds are from Ref. 9.
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]
[c] Mean from three determinations by a stopped-flow, CO_2 hydrase method.[19]

these two isozymes that are distinct from those observed for other cytosolic, mitochondrial or membrane-associated CAs investigated earlier.[7–12] Thus, for hCA VII, the following structure–activity relationship (SAR) was observed: (i) a consistent number of investigated derivatives, such as the aromatic amino acids **5**–**10**, histamine **11** and the structurally related aromatic/heterocyclic primary and secondary amines **14**–**18**, acted as weak hCA VII activators, with K_As in the range of 18.7–64.3 μM; (ii) medium potency hCA VII activating properties were observed for two investigated compounds, L- and D-Phe (compounds **3** and **4**), which showed K_As in the range of 9.74–10.93 μM, (iii) strong, submicromolar activating properties against hCA VII were observed on the other hand for the following compounds: L- and D-His (**1** and **2**), dopamine **12** and serotonin **13**, which showed K_As in the range of 0.71–0.93 μM. The best hCA VII activator was thus D-His **2**, and the most ineffective one in the small series of investigated compounds was 4-(2-aminoethyl)-morpholine **17**, the difference in activating properties between these two derivatives being 90.5-fold. SAR is thus rather simple for this small series of compounds: effective hCA VII activation is independent of the enantiomeric form of the amino acids. Best activators generally incorporated both amino and carboxy moieties, as well as a heterocyclic ring (imidazole, indole, etc.) or a substituted-aromatic one (3,4-dihydroxyphenyl, present in dopamine). There is also a striking difference of activity between the amino acid and the cognate amine obtained by its putative decarboxylation (for example L-/D-His and histamine; L-/D-DOPA and dopamine, respectively). Thus, in the first case, the amino acids **1** and **2** are much more efficient hCA VII activators as compared to histamine, whereas in the second one, dopamine **12** is more effective as compared to L-/D-DOPA **5** and **6**. Thus, effective activators of this isoform may be obtained both in the class of amines and amino acids.

The hCA XIV activating properties were strikingly different from what was shown above for the cytosolic isoform hCA VII. Thus, (i) the least efficient hCA XIV activators were D-DOPA **6**, D-Trp **8**, L-Tyr **9** as well as amines **14, 16** and **18**, which showed K_As in the range of 18.0–36.8 μM; (ii) more potent activating effects were observed with the following compounds: D-Phe **4**, L-DOPA **5**, L-Trp **7**, dopamine **12**, serotonin **13** as well as amines **15** and **17**, which showed K_As in the range of 5.4–16.5 μM (Table 2); (iii) the best hCA XIV activators in the investigated series of amino acids/amines were: L-/D-His (**1** and **2**), L-Phe (**3**), 4-amino-phenylalanine **10** and histamine **11**, with K_As in the range of 10 nM–2.90 μM. It may be observed that the most efficient hCA XIV activator is histamine, which shows a low nanomolar affinity for this isozyme (K_A of 10 nM), whereas the worst one is D-DOPA, which with an affinity of 36.8 μM is a 3680-fold less effective hCA XIV activator as compared to histamine. Thus, the range in activating properties for this series of derivatives is much wider in the case of hCA XIV (3680-fold) as compared to hCA VII (90-fold, see above). SAR is different here as compared to isoform hCA VII: L-amino acids are better hCA XIV activators as compared to the corresponding D-enantiomers. Furthermore, such effective activators incorporate again a heterocyclic ring or a substituted benzene one (such as in derivatives **1**–**3, 10, 11, 13, 15** and **17**).

The data in Table 2 also show that the newly investigated isoforms hCA VII and XIV possess activation profiles quite distinct from any other CA isozyme investigated earlier.[1,6–12] This is particularly true for hCA I and II which are ubiquitously present in many tissues in mammals, where they perform house-keeping functions mainly related to the acid–base homeostasis, transport of bicarbonate and secretion of electrolytes.[1–4] Thus, histamine **11** for example acts as a very efficient hCA XIV activator (K_A of 10 nM), being a medium potency hCA I activator (K_A of 2.1 μM) but a quite inefficient hCA II and hCA VII activator (K_As in the range of 37.5–125 μM). Thus, histamine might be considered a hCA XIV-selective CAA, although it appreciably activates some other isoforms too.[6–12] D-His **2** on the other hand acts as an efficient hCA I and VII activator (K_As in the range of 90 nM–0.71 μM), being slightly less efficient as hCA XIV activator (K_A of 2.37 μM) and much less inefficient as hCA II activator (K_A of 43 μM). All these data show that specific activation profiles of certain CA isozymes can be revealed already with the small set of compounds investigated here. This kind of an approach might constitute an interesting tool for a better understanding of the physiological functions of these enzymes.

Many of the investigated compounds such as the amino acids L-His, L-Phe, L-DOPA, L-Trp, L-Tyr, or the amines histamine, dopamine, serotonin or L-adrenaline, are present at a high enough concentration in various human tissues, including the brain,[21,22] and might thus act as endogenous activators for some of the CA isozymes present there, leading thus to a notable enhancement of their catalytic properties. This is likely to have important yet unravelled physiologic consequences, and it might be also useful when designing pharmacological agents based on CAA actions, a field that has not been extensively investigated so far.[13]

In conclusion, we report here the first detailed study on hCA VII and XIV activation based on a small library of natural and non-natural amino acids as well as aromatic/heterocyclic amines. hCA VII was activated efficiently by L- and D-His, dopamine and serotonin (K_As of 0.71–0.93 μM), whereas the best hCA XIV activators were histamine (K_A of 10 nM) and L-Phe, L-His, D-His and 4-amino-L-Phe (K_As of 0.24–2.90 μM). This study indicates that precise steric and electronic properties are needed for a molecule to act as an effective hCA VII or hCA XIV activator, which requires an adequate fit within the enzyme active site cavity for the formation of the enzyme-activator complex, and for an efficient proton transfer process within this complex that leads to the release

of a proton and formation of the catalytically active, zinc-hydroxide species of the enzyme. The present data may be useful as a starting point in the design of pharmacologic agents for the management of various kinds of brain disorders, including epilepsy and Alzheimer's disease.

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References and notes

1. Briganti, F.; Mangani, S.; Orioli, P.; Scozzafava, A.; Vernaglione, G.; Supuran, C. T. *Biochemistry* **1997**, *36*, 10384.
2. (a) Pastorekova, S.; Parkkila, S.; Pastorek, J.; Supuran, C. T *J. Enzyme Inhib. Med. Chem.* **2004**, *19*, 199; (b) Scozzafava, A.; Mastrolorenzo, A.; Supuran, C. T. *Exp. Opin. Ther. Pat.* **2004**, *14*, 667; (c) Supuran, C. T.; Scozzafava, A.; Casini, A. *Med. Res. Rev.* **2003**, *23*, 146; (d) Scozzafava, A.; Mastrolorenzo, A.; Supuran, C. T. *Exp. Opin. Ther. Pat* **2006**, *16*, 1627.
3. (a) Ilies, M.; Scozzafava, A.; Supuran, C. T. Carbonic anhydrase activators. In *Carbonic anhydrase – Its inhibitors and activators*; Supuran, C. T., Scozzafava, A., Conway, J., Eds.; CRC Press: Boca Raton (FL), USA, 2004; p 317; (b) Supuran, C. T.; Scozzafava, A. Activation of carbonic anhydrase isozymes. In *The Carbonic Anhydrases – New Horizons*; Chegwidden, W. R., Carter, N., Edwards, Y., Eds.; Birkhauser Verlag: Basel, Switzerland, 2000; p 197; (c) Supuran, C. T. *Therapy* **2007**, *4*, 355.
4. (a) Christianson, D. W.; Fierke, C. A. *Acc. Chem. Res* **1996**, *29*, 331; (b) Whittington, D. A.; Grubb, J. H.; Waheed, A.; Shah, G. N.; Sly, W. S.; Christianson, D. W. *J. Biol. Chem* **2004**, *279*, 7223; (c)*Carbonic Anhydrase – Its Inhibitors and Activators*; Supuran, C. T., Scozzafava, A., Conway, J., Eds.; CRC Press: Boca Raton, New York, London, 2004; p 1; (d) Nair, S. K.; Christianson, D. W. *J. Am. Chem. Soc.* **1991**, *113*, 9455.
5. (a) Supuran, C. T. Carbonic anhydrases: catalytic and inhibition mechanisms, distribution and physiological roles. In *Carbonic anhydrase – Its inhibitors and activators*; Supuran, C. T., Scozzafava, A., Conway, J., Eds.; CRC Press: Boca Raton, 2004; p 1; (b) Supuran, C. T.; Scozzafava, A.; Casini, A. Development of sulfonamide carbonic anhydrase inhibitors. In *Carbonic anhydrase – Its inhibitors and activators*; Supuran, C. T., Scozzafava, A., Conway, J., Eds.; CRC Press: Boca Raton, 2004; p 67; (c) Winum, J. Y.; Montero, J. L.; Scozzafava, A.; Supuran, C. T. *Mini-Rev. Med. Chem.* **2006**, *6*, 921.
6. Temperini, C.; Scozzafava, A.; Puccetti, L.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 5136.
7. (a) Temperini, C.; Scozzafava, A.; Vullo, D.; Supuran, C. T. *Chemistry* **2006**, *12*, 7057; (b) Temperini, C.; Scozzafava, A.; Vullo, D.; Supuran, C. T. *J. Med. Chem.* **2006**, *49*, 3019.
8. (a) Temperini, C.; Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 5152; (b) Temperini, C.; Innocenti, A.; Scozzafava, A.; Mastrolorenzo, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 628.
9. Parkkila, S.; Vullo, D.; Puccetti, L.; Parkkila, A. K.; Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 3955.
10. (a) Vullo, D.; Nishimori, I.; Innocenti, A.; Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 1336; (b) Nishimori, I.; Vullo, D.; Innocenti, A.; Scozzafava, A.; Supuran, C.T. *Bioorg. Med. Chem.* **2007**, *15*, in press. doi:10.1016/j.bmc.2007.03.004.
11. Scozzafava, A.; Supuran, C. T. *J. Med. Chem.* **2002**, *45*, 284; (b) Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 1177.
12. (a) Ilies, M.; Banciu, M. D.; Ilies, M. A.; Scozzafava, A.; Caproiu, M. T.; Supuran, C. T. *J. Med. Chem.* **2002**, *45*, 504; (b) Scozzafava, A.; Iorga, B.; Supuran, C. T. *J. Enzyme Inhib.* **2000**, *15*, 139.
13. (a) Sun, M-K.; Alkon, D. L. *Trends Pharmacol. Sci.* **2002**, *23*, 83; (b) Sun, M. K.; Alkon, D. L. *J. Pharmacol. Exp. Ther.* **2001**, *297*, 961.
14. Lakkis, M. M.; O'Shea, K. S.; Tashian, R. E. *J. Histochem. Cytochem.* **1997**, *45*, 657.
15. (a) Ruusuvuori, E.; Li, H.; Huttu, K.; Palva, J. M.; Smirnov, S.; Rivera, C.; Kaila, K.; Voipio, J. *J. Neurosci.* **2004**, *24*, 2699; (b) Rivera, C.; Voipio, J.; Kaila, K. *J. Physiol.* **2005**, *562*, 27.
16. (a) Vullo, D.; Voipio, J.; Innocenti, A.; Rivera, C.; Ranki, H.; Scozzafava, A.; Kaila, K.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 971; (b) Winum, J. Y.; Scozzafava, A.; Montero, J.-L.; Supuran, C. T. . *Med. Res. Rev.* **2005**, *25*, 186.
17. (a) Kaunisto, K.; Parkkila, S.; Rajaniemi, H.; Waheed, A.; Grubb, J.; Sly, W. S. *Kidney Int.* **2002**, *61*, 2111; (b) Parkkila, S.; Parkkila, A.-K.; Rajaniemi, H.; Shah, N. G.; Grubb, J. H.; Waheed, A.; Sly, W. S. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 1918; (c) Parkkila, S.; Kivelä, A. J.; Kaunisto, K.; Parkkila, A.-K.; Hakkola, J.; Waheed, A.; Sly, W. S.; Rajaniemi, H. *BMC Gastroenterol.* **2002**, *2*, 13.
18. (a) Nishimori, I.; Vullo, D.; Innocenti, A.; Scozzafava, A.; Mastrolorenzo, A.; Supuran, C. T. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 3828; (b) Özensoy, Ö.; Nishimori, I.; Vullo, D.; Puccetti, L.; Scozzafava, A.; Supuran, C. T. *Bioorg. Med. Chem.* **2005**, *13*, 6089.
19. 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. 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 ($[S] \gg K_M$), 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]

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

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

20. Liljas, A.; Hakansson, K.; Jonsson, B. H.; Xue, Y. *Eur. J. Biochem.* **1994**, *219*, 1.
21. Izumi, M.; Yamazaki, H.; Nakabayashi, H.; Owada, M. *No To Hattatsu* **2006**, *38*, 27.
22. Torres, N.; Beristain, L.; Bourges, H.; Tovar, A. R. *J. Nutr.* **1999**, *129*, 1979.