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AMINO ACID HYDROXAMATES AS INHIBITORS OF THE HUMAN ENKEPHALIN-DEGRADING AMINOPEPTIDASE

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Amino acid hydroxamate derivatives inhibit the recently characterized enkephalin-degrading aminopeptidase from human blood (α-aminoacyl-peptide hydrolase, EC 3.4.11.11). The efficiency of inhibition depends on the structure of the amino acid hydroxamate employed. Amino acid hydroxamate derivatives also inhibit metalloendopeptidases and enkephalin-degrading enzymes from rat brain. The degradation of enkephalin in blood and in the brain seems to be under the control of a number of metallopeptidases: suitable amino acid hydroxamate derivatives can therefore be proposed as general inhibitors of enkephalin breakdown.

INTRODUCTION

Blood contains a highly effective deactivation system for opioid peptides, enkephalins (1). The principal enzyme involved in this process, which has recently been purified and characterized (2), is a soluble aminopeptidase which cleaves the Tyr^1-Gly^2 bond of enkephalins and belongs to the class of metallopeptidases (3). This group of enzymes is involved in many important physiological processes including digestion (carboxy-peptidases), regulation of blood pressure (angiotensin-converting enzyme) and tissue remodelling (collagenases). Several natural or synthetic inhibitors of metallopeptidases are known and have been used in studies on the regulation of the activity of these enzymes (4,5,6).

Aminopeptidase from human blood hydrolyses enkephalins and less specifically the first peptide bond of short peptides when the first amino acid is aromatic (2). It determines the transient half-life of enkephalins in circulating blood, which might account for the limited potency of this peptide when given intra-venously. To study the contribution of this enzyme activity to the regulation of enkephalin levels and the possibility of modifying the pharmacological behaviour of opioid peptides by altering their metabolism, we investigated the inhibitory power of a number of compounds. Structurally, efficient competitive inhibitors should comprise at least two groups of high affinity, one of them designed to bind as the substrate and the other to interact with the catalytic site of the enzyme (7). In the case

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of amino-enkephalinase, the C-derivation of an aromatic amino acid (substrate site) to make it a chelating reagent (metal-ion active site) should produce an efficient inhibitor of the enzyme. In this paper we report that hydroxamate derivatives fulfill the above parameters and are efficient inhibitors.

EXPERIMENTAL

Leu- enkephalin was purchased from Bachem A.G. (Bubendorf, Switzerland). and the (L)-amino acid hydroxamates from Sigma Chemical Co. (St. Louis, MO). When required, the other hydroxamate derivatives were synthetized by hydroxylaminolysis of the corresponding ester derivatives. Other chemicals were of the purest commercial grade available products.

Enzyme preparation. Human plasma was treated with ammonium sulfate and chromatographed on DEAE-Sephadex, 5 mM phosphate buffer, from pH 7.8 to pH 7.5 (0.5 M NaCl). The following steps of the purification were as described previously (2,3).

Determination of the enzyme activity. Leu-enkephalin was treated with the amino-enkephalinase at 37°C, pH 7.8 for suitable lengths of time and the activity was followed by quantitative determination of the released N-terminal tyrosine as a function of time on a Beckman Amino Acid Analyser Model 119 C, as described elsewhere (2). Inhibitors were added to the enzyme solutions 10 min before the addition of enkephalin. The reaction was stopped by addition of the amino acid analyser buffer, pH 2.2. The inhibition values were determined by Dixon plots employing 0.4, 0.8 and 1.2 mM substrate concentrations.

RESULTS AND DISCUSSION

Thiol compounds were analysed since they have been proposed as inhibitors of metalloendopeptidases (8) and, more recently, of brain enkephalinase (9). In the case of human blood aminopeptidase, mercaptoethanol, thiophenol and 4-NH_2-thiophenol were tested and at a concentration of 10 mM they inhibited the enzyme activity of respectively 15%, 37% and 65%. Sulfur containing peptides, such as Tyr-CySH and Tyr-Gly-CySH, were also ineffective. Thiol compounds are thus poor inhibitors of human blood amino-enkephalinase and must be discarded for the study of this enzyme. It should be noticed that, since they are strong inhibitors of brain enkephalinase (9), they might be useful to differentiate the impact of these enzymatic activities "in vivo".

Hydroxamate groups have shown to be very efficient at inactivating the human amino-enkephalinase, probably because of a specific interaction with the metal-ion active site of the enzyme. The K_i app. values of (L)-amino acid hydroxamates are listed in Table. It can be seen that, as expected, the aromatic derivatives are the strongest inhibitors of the enzyme. However, a lower but still significant inhibition was observed with aliphatic (Ala) or basic (Arg) amino acid derivatives. The inhibition is competitive, as exemplified by the Lineweaver-Burk plots shown in Figure, and is totally reversible.

In order to clarify the relationship between the functional groups of the amino acid hydroxamate and their inhibitory power, a number of derivatives

TABLE

Inhibition of amino-enkephalinase by amino acid hydroxamates

R	K_i of X-NH-CH-CO-Y (R)		
	X = H, Y = NHOH	X = H, Y = OMe or NH_2	X = CHO, Y = NHOH
Tryptophan	3×10^{-6} M	$\gg 10^{-2}$ M	$\gg 10^{-2}$ M
Tyrosine	4×10^{-5} M	$\gg 10^{-2}$ M	$\gg 10^{-2}$ M
Phenylalanine	5×10^{-5} M	n.d.	n.d.
Arginine	1×10^{-4} M	negligible inhibition	
Alanine	3×10^{-4} M	negligible inhibition	

Activity was determined with Leu-enkephalin as described under Experimental.

conveniently modified at the hydroxamic acid function and/or at the N-terminal group were examined and the resulting effects on the amino-enkephalinase activity are summarized in Table. The results suggest that, apart from the effect of the structure of the amino acid moiety itself, the NH_2-terminal group must be preserved to maintain the inhibitory power. Since the same behaviour was observed with substrates whose susceptibility to digestion is completely suppressed in des-NH_2 peptides (2,3), this group must be considered critical for the efficient binding of the enzyme to both substrates and inhibitors. Hydroxamate group is also crucial for inhibition as shown by the dramatic decrease in inhibitory potency of other carboxy-substituted aromatic amino acids. The best inhibitor so far evidentiated is, as shown in Table, (L)-tryptophan hydroxamate. For some of the derivatives the (D)-isomers were tested but the difference in K_i values were too small to postulate a distinct stereospecificity.

Amino acid and/or peptide hydroxamates are inhibitors of metalloendopeptidases, such as thermolysin (10,11), but not of some metalloexopeptidases, such as Leu-aminopeptidase (12). Thus the specificity of their action can be proposed as a parameter for distinguishing the human amino-enkephalinase degrading action from less specific aminopeptidase actions in the study of biological samples.

Brain particulate enkephalinase, which was initially characterized as a dipeptidyl-carboxypeptidase (13) but has more recently (14,15) been described

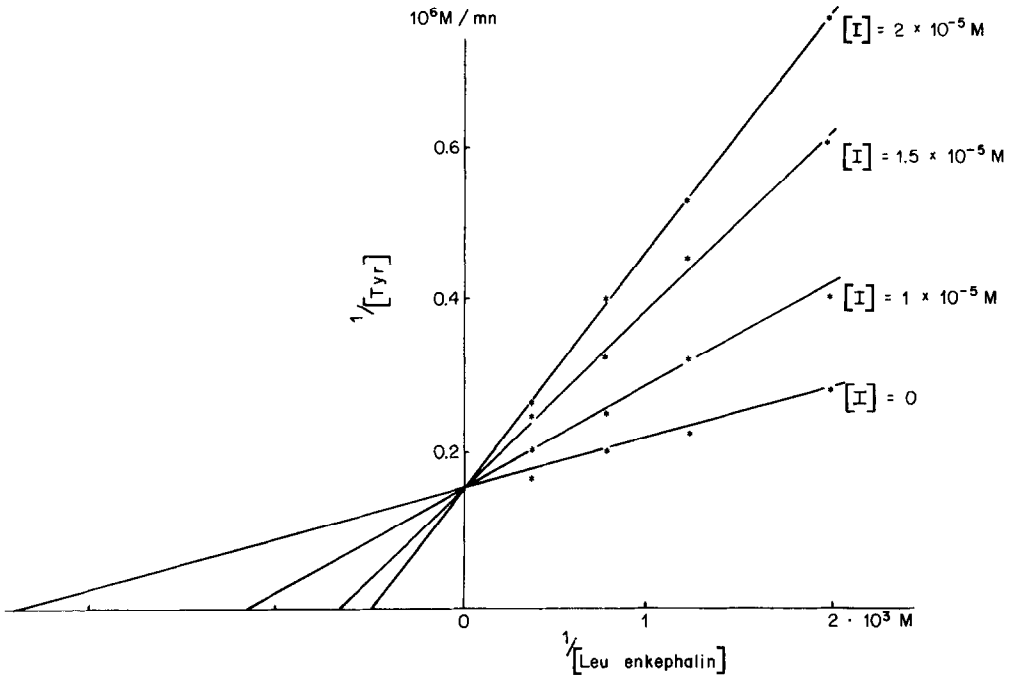

Figure Lineweaver-Burk plots of the tyrosine liberated from Leu-enkephalin by aminoenkephalinase (0.5×10^{-8} M), pH 7.8, in absence and in presence of increasing amounts of (L)-tryptophan hydroxamate (I).

as an endopeptidase similar to thermolysin, is also strongly inhibited by amino acid hydroxamates (16). Inhibition of the brain amino-enkephalinase has also been reported (16,17) but in contrast to the resuts presented here, the N-terminal group can apparently be blocked in inhibitors of the brain enzyme while it must be free for efficient inhibition of the blood enzyme (see Table). In spite of these minor differences amino acid hydroxamates, especially the aromatic ones, can thus be considered as general inhibitors of enkephalin breakdown both in circulating blood and in the brain and will be useful for investigating the relationships between opioid peptides and analgesia.

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