'In vivo' amplification of biological activity of tetragastrin by amino acid hydroxamates

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(Received 20 November 1984)

Rat blood was shown to contain an aminopeptidase which rapidly hydrolyses short peptides containing an aromatic amino acid as N-terminal residue. Using tetragastrin (Trp-Met-Asp-PheNH₂) as substrate, we showed that some amino acid hydroxamates inhibit rat aminopeptidase activity 'in vitro' in the following order: HTrpNHOH > HPheNHOH >> HAlaNHOH. The same hydroxamates markedly enhanced the biological activity of tetragastrin 'in vivo'. The amplification of the secretory effect, correlated with the amount of the hydroxamate used, strongly suggests that these compounds can stabilize a number of active peptides in vivo by inhibiting their proteolytic degradation.

The blood of vertebrates contains a potent proteolytic activity (1,2) which is responsible for the transient half-life of several peptides in circulating fluids. The enzyme from human blood was purified (2) and characterized: it was shown to be an aminopeptidase which cleaves the N-terminal residues of peptides containing 3 to 12 amino acid residues and beginning with an aromatic amino acid. This enzyme proved to be a metallo-protease (3), and inhibitors containing appropriate structural features were designed and synthesized (4,5): the best in vitro inhibitor is HTrpNHOH (Ki = 3.7 x 10⁻⁶ M).

The gastrin-related compounds constitute a family of biologically active peptides which contains the same oligopeptide fragment, located on the carboxyl end. The tetrapeptide amide, Trp-Met-Asp-PheNH₂, usually called 'tetragastrin', is the smallest fragment possessing a full gastric secretory activity. When these peptides are viewed as substrates for the aminopeptidase described above, they should exhibit different stabilities to proteolytic degradation because of their differences in the length of the peptide chain and in the N-terminal amino acid structure. Among these peptides, tetragastrin is the only one which completely loses its biological activity by removing its N-terminal tryptophan residue (6).

The aim of this study was to investigate on one hand the inhibition of the aminopeptidase activity 'in vivo' and its potential pharmacological interest, and on the other hand the consequence of...
aminopeptidase degradation of gastrin-active peptides on their biological activity. For this purpose, we studied the effect of amino acid hydroxamates on the stimulation of gastric acid secretion induced by tetragastrin when administered intravenously to rats. This model was shown to be reliable and highly sensitive with respect to gastrin and its analogues. In addition, the aminopeptidase from rat blood has been partially characterized with special attention to its action on tetragastrin and its inhibition by amino acid hydroxamates 'in vitro'.

Materials and Methods

$N^\alpha$-formyl peptide

Tetragastrin (50 mg) was dissolved in formic acid (1 ml) and treated with acetic anhydride (0.3 ml) (7). The reaction mixture was allowed to stand for 2 h at room temperature and then was diluted with water (10 ml): three extractions (3 ml each) with n-butanol were performed on the aqueous solution. Organic layers were collected and evaporated under reduced pressure. Residues were dissolved in the minimum amount of 0.2-M $\text{NH}_2\text{OH}$ and purified by chromatography on a column (30 x 2.5 cm) of Sephadex LH20 equilibrated in the same solvent. $N^\alpha$-formyl-tetragastrin arises as the principal product and after lyophilization, 30 mg of a white, fluffy product was obtained.

Enzyme purification

Heparinized blood from male rats was centrifuged and dialysed overnight. Part of the resulting solution (total plasma) was submitted to anion-exchange chromatography and molecular sieving, as described for the purification of the human enzyme (2). The final sample was 15-fold enriched in enzyme (aminopeptidase) and devoid of other proteolytic activities.

Enzyme assay

The aminopeptidase activity of total plasma and of purified enzyme was measured by following the release of free tryptophan from tetragastrin either by amino acid analysis on a Beckman model 119C or by h.p.l.c. on a Beckman automated gradient system model 330. Peptides were separated on an Ultrasphere O.D.S. (C18) column with an increasing linear gradient of acetonitrile in phosphoric acid-triethylamine buffer, pH 3, and were detected by u.v. at 254 nm. The identification of the hydrolysis products was made by comparison with authentic samples. The kinetic parameters were calculated from Lineweaver-Burk plots.

Inhibitors assay

The inhibitory effect of HTrpNHOH, HPheNHOH, and HAlaNHOH (Sigma Chemicals, U.S.A.) on the enzyme activity was measured both on purified enzyme (Ki) as described elsewhere (5) and on total plasma.
Bioassay

Gastric acid secretion induced by gastrin or its analogues was determined on an anaesthetized rat according to the experimental procedure of Ghosh, which was modified (8) as follows: fasted male rats (300 g) were anaesthetized with urethane; after tracheotomy and venous catheterization, the oesophagus and duodenum were cannulated. A closed loop with a propionate-succinate solution was established through the stomach with a constant rate pump of 3 ml/min. Body and solution were kept at 30°C. Acid secretion from the stomach induced a pH decrease detectable by a glass electrode and monitored as a function of time. After stabilization of basal acid secretion, gastrin or related peptides were injected i.v. and the response was recorded for 40 min. In the range from pH 5.5 to pH 3, this propionate-succinate solution gave a linear pH variation with H+ concentration. The pH difference between the maximal value and the value extrapolated from the basal line at the same time was measured on the tracing. Results are expressed as micromoles of H+ secreted in response to the peptide administration.

Results and Discussion

When the tetrapeptide amide Trp-Met-Asp-PheNH₂ is treated in vitro with rat serum, it rapidly disappears mainly as a consequence of the cleavage of the first peptide bond with a release of free tryptophan due to an aminopeptidase activity. The same tetrapeptide amide became much more stable under the same conditions when its terminal amino group was blocked by acylation (Fig. 1). In our study, we used

![Graph](image-url)  
**Fig. 1.** Behaviour of tetragastrin and N²-formyl-tetragastrin in rat plasma. Peptides are incubated in plasma solution (20% v/v in 50-mM phosphate buffer, pH 7.8) at 37°C.
Nα-formyl-tetragastrin as N-blocked peptide because of its solubility in aqueous solutions and its elevated biological activity.

The specific activity of the aminopeptidase from rat serum was about twice as high (× 2.3) as that from human serum. The enzyme exhibits a maximum activity between pH 7.5 and 8.3. The Lineweaver-Burk plot of enzyme activity using tetragastrin as substrate is reported in Fig. 2 with an apparent Michaelis constant of 1.25 mM. The enzyme isolated from rat serum shares some characteristics with the human one: the mol. wt. is about 85 000 (from Sephadex G-100 gel filtration) and short peptides (3 to 10 amino acids) are susceptible substrates if their N-terminal amino acid is an aromatic one. When the N-terminal amino group of the peptides is masked (i.e. by formylation) or removed (i.e. desamino-peptides), they lose their susceptibility to the enzyme action.

As pointed out for the human enzyme (3) the rat aminopeptidase activity was reduced by metal chelators such as o-phenanthroline. Another class of enzyme inhibitors with in vitro chelating properties (4) are the hydroxamate derivatives of aromatic amino acids. The inhibition constants (Ki), determined on the purified rat enzyme, were $3.7 \times 10^{-6}$ M for the tryptophan derivative and $29 \times 10^{-6}$ M for the phenylalanine one. Hydroxamates from aliphatic amino acids inhibited the enzyme to a lesser extent (Ki for HAlaNHOH = $200 \times 10^{-6}$ M). If we examine the concentration required to inhibit the enzyme activity in total plasma to 50% (IC-50), the values were higher than that needed for the purified enzyme, probably because part of the

![Fig. 2. Lineweaver-Burk plot of the enzyme activity from rat plasma. The substrate was tetragastrin. The release of tryptophan was followed by amino acid analysis for each substrate concentration.](image-url)
Fig. 3. Comparative IC-50 determinations of the inhibitory effect of amino acid hydroxamates on the rat blood enzyme activity. The enzymatic residual activity (%) (37°C, 15 min) was plotted as a function of amino acid hydroxamate concentration. (△) HTrpNHOH, (◆) HPheNHOH, (□) HAlaNHOH.

hydroxamate was subtracted for other competitive linkages. Nevertheless, the order of inhibitory potency of the different hydroxamates (Fig. 3) was maintained (HAlaNHOH was 7 times and HPheNHOH 1.6 times less potent than HTrpNHOH).

Table 1 summarizes the results of biological activities of both tetragastrin and its formylated active derivative (N0α-formyl-tetragastrin), and the differential effects of amino acid hydroxamates on these activities. Amino acid hydroxamates intravenously administered together with the active peptide display different effects depending on whether the peptide possesses or does not possess a free terminal amino group: the biological activity of tetragastrin was markedly amplified by aromatic amino acid hydroxamates while the activity of N0α-formyl-tetragastrin remains unchanged when examined under the same experimental conditions. The extent of this amplificatory effect parallels the inhibitory potency on serum aminopeptidase as reported in Fig. 3. The sensitivity of tetragastrin together with the contrasting stability of N0α-formyl-tetragastrin in respect to the blood aminopeptidase activity (Fig. 1) strongly suggested that amino acid hydroxamates inhibit tetragastrin degradation both in vitro and in vivo. Incidentally, it is noteworthy that the N0α-formylation of tetragastrin yielded a compound easily obtained and slightly more potent than the currently used pentagastrin (Boc-β-Ala-Trp-Met-Asp-PheNH2) (6).
Table 1. Secretory activity of tetragastrin and Nα-formyl-tetragastrin in the presence of amino acid hydroxamates in the anaesthetized rat (Ghosh)

Peptides (in 0.01-N NH₄OH) were injected intravenously in a small volume (20 μl) alone or after bolus injection of various doses of hydroxamates. The response was measured (in μmol H⁺) after stabilization of the secretion and compared to that of Synthetic Human Gastrin I (G-17) (gift from Dr. L. Moroder, Max Planck Institut, Munchen, RFA) as standard.

<table>
<thead>
<tr>
<th>Hydroxamate (mg/kg)</th>
<th>Tetragastrin (3 μg/kg)</th>
<th>Nα-formyl tetragastrin (2.5 μg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>3 ± 1.5b</td>
<td>21 ± 3b</td>
</tr>
<tr>
<td>HTrpNHOH (0.16)</td>
<td>12 ± 2c</td>
<td>24 ± 4c</td>
</tr>
<tr>
<td>(3.25)</td>
<td>16 ± 2c</td>
<td>22 ± 4c</td>
</tr>
<tr>
<td>(10.0)</td>
<td>20 ± 2c</td>
<td>22 ± 4c</td>
</tr>
<tr>
<td>HPhεNHOH (0.16)</td>
<td>10 ± 3c</td>
<td>23 ± 3c</td>
</tr>
<tr>
<td>HAlaNHOH (10.0)</td>
<td>6 ± 3c</td>
<td>23 ± 3c</td>
</tr>
</tbody>
</table>

*Using G-17 as internal standard (0.5 μg/kg, i.v.) on the same model, we obtained, over 17 experiments, 20 ± 3 μmol H⁺.

a 5 experiments.

Other in vivo degradative processes for pentagastrin have been described, namely its extraction from plasma by the liver (9,10). The aminopeptidase-mediated degradation is an additive process which occurs with peptides presenting the structural features described above. With regard to the structure-activity relationships in the field of gastrin-related peptides, tetragastrin is the peptide which disappears from the circulation at a much higher rate than does its acyl derivative or any other gastrin fragment with a non-aromatic amino acid residue in the N-terminal position and/or a higher molecular weight. Under hydroxamate protection, tetragastrin recovers its real 'intrinsic' activity and this must be taken into account when the structural requirements involved in the binding to receptors are studied.

Very little is known about the toxicity of amino acid hydroxamates (11). We were unable to detect any visible effect on acutely treated rats using doses up to 30 mg/kg. The development of an effective treatment for hypertension based on specific inhibition of the angiotensin-converting enzyme (12,13) has aroused great interest in the potential applications of effective and specific inhibitors of peptide-degrading enzymes.

Besides diagnostic and/or therapeutic applications which need further development, amino acid hydroxamates can be considered good candidates for studies on the metabolism of peptides which possess free terminal amino groups and on the impact of degradative dynamic processes that control the availability of biologically active peptides.
References