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INHIBITORY POTENCY OF VARIOUS PEPTIDES ON ENKEPHALINASE ACTIVITY FROM MOUSE STRIATUM

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SUMMARY :

A variety of peptides chemically unrelated to enkephalins are relatively good inhibitors (IC_{50} in the micromolar range) of "enkephalinase" activity i.e. of the peptidase releasing Tyr-Gly-Gly from Leu-enkephalin. Its specificity has been also reinvestigated with a series of Met-enkephalin analogues. The poor recognition of several analogues by this inactivating enzyme might account for their enhanced biological activity.

The endogenous opioid pentapeptides Met-enkephalin (Met-ENK i.e. Tyr-Gly-Gly-Phe-Met) and Leu-enkephalin (Leu-ENK i.e. Tyr-Gly-Gly-Phe-Leu) are subject to extremely rapid inactivation in brain tissues. In the presence of brain homogenates, this occurs mainly by cleavage of the Tyr-Gly amide bond (1-6). In addition we have recently demonstrated that a membrane-bound enzyme in rodent brain releases the tripeptide Tyr-Gly-Gly from the ENKs (7,8,9). This "enkephalinase" activity might be selectively involved in turning-off the ENK signal in nervous tissue, as mainly suggested by its regional distribution paralleling that of opiate receptors (8) and by its increase following administration of morphine (7). Enkephalinase, a dipeptidyl carboxypeptidase, resembles angiotensin-converting enzyme (ACE ; EC 3.4.15.1) an enzyme of broad substrate specificity also present in brain (10) and also able to cleave the Gly-Phe bond of ENKs. Although both enzymes appear to contain Zn in their catalytic site (11,12) a large body of evidence indicates that enkephalinase is distinct from ACE : it is not activated by Cl^- ions ; its regional distribution and developmental pattern during ontogenesis differ markedly from those of ACE ; it is inhibited by only relatively high concentrations of potent ACE inhibitors like SQ 14,225, wich exhibits a 1,000 fold difference in Ki for the two enzymes; it is activated following morphine treatments, whereas ACE is not (12,13).

From the large differences in inhibitory potencies of a variety of peptides, it was originally concluded that enkephalinase has a narrow subs-

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trate specificity and a very high affinity for the two ENKs (7). However these conclusions were reached by analysis of biphasic saturation curves of overall ENK-hydrolysing activity, in which enkephalinase activity only represented even at low substrate concentration approximately 20% of the total. Due to interference of the aminopeptidase activity this method for enkephalinase assay was of low accuracy and did not allow to determine the inhibitory potency of several peptides (7).

Because puromycin is a potent inhibitor of aminopeptidase activity (14, 15,16,7) but not of enkephalinase whereas the reverse is true for the tetrapeptide Gly-Gly-Phe-Met, these compounds can be utilised for a simple and selective assay of enkephalinase activity (8). We have now used this assay to reinvestigate the specificity of this enzyme by determining the inhibitory potency of a variety of natural peptides and of fragments or derivatives of the ENK molecule.

MATERIALS AND METHODS.

Preparation of the particulate fraction.

The striata of three male Swiss mice were homogenized into 10 µl of cold 0.05 M Tris-HCl buffer, pH 7.4 (3 x 5 sec at 1,500 rpm in a glass-teflon Potter homogenizer, clearance 0.1-0.15 mm). The particulate fraction was obtained by two successive centrifugations (10^3g x min and 2.10^5g x min), washed by resuspension into 10 ml of cold buffer, followed by a new centrifugation at 2.10^5g x min. The resulting pellet was resuspended in 7 ml of buffer with a Dounce Homogenizer (clearance 20-55 µM).

Enzyme assays.

A 50 µl sample of the particulate fraction (approximately 0.5 mg protein ml^{-1}) was preincubated for 15 min at 25°C. Incubations (final volume 0.1 ml) were performed at the same temperature during 15 min in the presence of 10 nM ^{3}H-Leu-ENK (39 Ci-mmol^{-1}, the Radiochemical Centre, Amersham, purified before the experiment by Porapak column chromatography) and 0.1 mM puromycin, an aminopeptidase inhibitor, alone or in the presence of 0.1 mM Gly-Gly-Phe-Met, a selective inhibitor of enkephalinase (8). The incubations were stopped by the addition of 25 µl of 0.2 N HCl and ^{3}H-metabolites were separated from intact ^{3}H-Leu-ENK by Porapak column chromatography (3,8). For this purpose, a 50 µl sample was applied on 80 mg Porapak (100-120 mesh, Waters Assoc.) contained in a Pasteur pipette and the radioactivity eluted with 2 x 1 ml distilled water was quantified by liquid scintillation spectrometry. Enkephalinase activity i.e. ^{3}H-Tyr-Gly-Gly formation was evaluated as the difference between ^{3}H-metabolites formed in incubations performed in the absence and in the presence of 0.1 mM Gly-Gly-Phe-Met. The conditions ensured initial velocity reactions (less than 10% of substrate hydrolysed).

Thin-layer chromatography.

The validity of the column chromatography procedure used to estimate ^{3}H-Tyr-Gly-Gly formation was assessed by quantifying this tripeptide isolated by TLC. Samples of the incubation medium were applied on silica gel plates (plastic sheets, Merck, thickness 0.2 mm) and the chromatogram developed in the following solvent mixture : isopropanol ; AcOET ; 5% AcOH (2:2:1). Markers were visualised with a ninhydrin spray and radioactive peaks at their Rf were isolated by cutting the plates and counted by liquid scintillation spectrometry. Rf values were : Tyr = 0.49, Tyr-Gly-Gly = 0.25, Leu-ENK = 0.71.

Following a typical incubation in the presence of 0.1 mM puromycin but in the absence of Gly-Gly-Phe-Met, ^{3}H-Tyr represented 33% and ^{3}H-Tyr-Gly-Gly 67% of ^{3}H-metabolites formed. Enkephalinase activity evaluated either by TLC or Porapak chromatography of samples from the same incubations were always in excellent agreement, as shown for instance by data of table 1.

Determination of IC_{50} of various compounds.
Incubations were performed in the presence of 10 nM ^{3}H-Leu-ENK and increasing concentrations of the various compounds. Data anlysed according to Parker et Waud (17).

Chemical.
Puromycin was from Sigma (St Louis, USA). FK 33.824 was kindly donated by Sandoz (France). Dr Bajusz is gratefully acknowledged for the supply of D.Met^{2}-Pro^{5} ENKamide.

The listed peptides from Leu-ENK to Tyr-Gly-Gly-MePhe-Met (table 2) were synthetized by the liquid phase method using terbutyloxycarbonyl and methyl esters as protecting groups and dicyclohexyl carbodiimide with hydroxybenzotriazole as coupling reagents (18). Met-ENKamide and D.Ala^{2}-Met-ENK amide were obtained by direct reaction of the corresponding esters with ammonia followed by deprotection of the terbutyloxycarbonyl group by TFA (19). Other peptides were from commercial source (Sigma, USA and Bachem, Switzerland).

RESULTS AND DISCUSSION.

The Km values of Leu-ENK, 22 µM, (table 1) and the Ki value of Met-ENK 1.5 µM determined from the IC_{50} (table 2) assuming a competitive inhibition differ from the corresponding values already reported (7). As discussed before , this discrepancy can be explained by the poor accuracy of the previous enkephalinase assay technique which did not avoid interference of the high aminopeptidase activity.

From the data of table 2, it appears that enkephalinase does not exhibit an extremely stringent specificity towards a variety of peptides. This is evidenced by the IC_{50} in the micromolar range, like that of Met-ENK, of various peptides structurally unrelated to the ENK molecule. This is the case for relatively large peptides like insulin, as well as oligopeptides (angiotensin I, Gly-Gly-Phe-Met) or even dipeptides like Tyr-Gly. On the other hand a variety of other peptides, including β-endorphin, do not appear to be recognized

Table 1. Saturation kinetics of enkephalinase activity from mouse striatum using ^{3}H-Leu-ENK as substrate : comparison of two assay techniques.

Kinetic Parameters.	Porapak column Chromatography	Thin-layer Chromatography
V max (pmoles.min^{-1}.mg protein^{-1})	294 ± 47	281 ± 26
K m	2.2 ± 0.4 10^{-5}M	2.2 ± 0.3 10^{-5}M

Table 2. Inhibitory potency of various compounds on enkephalinase activity from mouse striatum.

Compounds	IC_{50} (μM)
Tyr-Gly-Gly-Phe-Leu OH (Leu-ENK)	11.0 ± 1.0
Tyr-Gly-Gly-Phe-Met OH (Met-ENK)	1.4 ± 0.5
Tyr-Gly-Gly-Phe-Met $\underline{OCH_3}$	8.3 ± 1.6
Tyr-Gly-Gly-Phe-Met $\underline{NH_2}$	40.0 ± 15
Tyr-D.Ala-Gly-Phe-Met OH	3.8 ± 0.7
Tyr-D.Ala-Gly-Phe-Met $\underline{NH_2}$	65.0 ± 8.0
Tyr-L.Ala-Gly-Phe-Met OH	2.3 ± 0.5
Tyr-Gly-L.Ala-Phe-Met OH	1.4 ± 0.4
Tyr-Gly-D.Ala-Phe-Met OH	32.0 ± 9.0
Tyr-Gly-Gly-D Phe-Met OH	11.0 ± 2.0
Tyr-Gly-Gly-MePhe-Met OH	44.0 ± 8.0
Tyr-D.Met-Gly-Phe-Pro $\underline{NH_2}$	310.0 ± 90
Tyr-D.Ala-Gly-MePhe-Met(O)-ol FK.33-824	420.0 ± 90
Tyr	> 100.0
Tyr-Gly	1.6 ± 0.9
Tyr-Arg	> 100.0
Tyr-D.Arg	> 100.0
Tyr-Lys	> 100.0
Gly-Gly-Phe-Met	1.7 ± 0.8
Glutathion (reduced form)	67.0 ± 4.0
Insulin (chain B)	4.7 ± 1.0
Carnosine	> 100.0
T.R.H.	> 100.0
L.H.R.H.	77.0 ± 38
β-endorphin (camel)	> 100.0
Angiotensin I	2.8 ± 1.5
Morphine	> 100.0

by the enzyme. It must be noticed that the technique used does not allow to conclude whether inhibitory peptides act as substrate or inhibitor of the enzyme. However we have checked that, at least in two cases i.e. insulin and Tyr-Gly the inhibition is of a competitive type (not shown).

This relatively low specificity of enkephalinase may suggest that the entire molecule is not necessarily recognized by the enzyme. However the determination of inhibitory potencies of ENK analogues throws more light on this

matter. The inversion of the configuration of the aminoacids either in position 3 (D.Ala$_3$-Met-ENK) or position 4 (D.Phe$_4$-Met-ENK) strongly reduces the inhibitory potency. Interestingly, although the effect is less marked when the inversion affects the aminoacid in position 2, it is still noticeable as shown by the significantly reduced potency of D.Ala$_2$-Met-ENK as compared to Met-ENK.

The importance of a free carboxyle group is nicely shown by the reduced potency when this group is esterified and even more when amidified, suggesting its interaction with the active site of enkephalinase. Assuming for this dipeptidyl carboxypeptidase a catalytic mechanism analogous to that of carboxypeptidase A (20), this feature could be expected. A similar assumption could explain the low potency of Tyr-Arg and Tyr-Lys as compared to Tyr-Gly : in carboxypeptidase A the free carboxyle group interacts with Arg an effect which is conceivably inhibited when the C-terminal aminoacid of the peptide is of basic nature . The low potency of Tyr-L-Arg and Tyr-D-Arg excludes that inhibition of enkephalinase accounts for their analgesic activity (21). On the other hand the good inhibitory potency of dipeptides like Tyr-Gly might explain that a mixture of dipeptides could protect endogenous ENKs released from depolarised slices by inhibiting enkephalinase (22).

The loss of potency in Tyr-Gly-Gly-MePhe-Met could be due either to a steric hindrance by the methyl group in the vicinity of the amide bond to be cleaved or to the loss of an eventual hydrogen-bonded interaction with the enzyme at this level.

Taken together these features may account for the increased biological activity (not explained by a higher affinity for receptors or resistance to aminopeptidase activity) in ENK analogues combining the various modifications resulting in a lesser recognition by enkephalinase. This is the case of Tyr-D-Met-Gly-Phe-Pro-NH_2 (23) a compound 20-times more active as an analgesic than Met-ENK (25) and even more for FK 33-824 (24) which is 250-times more potent (25). In connection with this, it is interesting to observe that the inhibition of aminopeptidase activity by replacement of Gly^2 by $D.Ala^2$ in ENKs leads to a three times increase of the analgesic activity whereas the protection from enkephalinase activity by methylation of the Gly-Phe peptide bond produces a twelve times enhancement of potency (25).

In conclusion, our data indicate that enkephalinase exhibits a somewhat lower specificity than previously suggested. However this does not exclude a physiological role in the inactivation of endogenous ENKs, if this enzyme is strategically located in the vicinity of opiate receptors. In any event the poor recognition of several ENK analogues by enkephalinase could explain, at least in part, their enhanced biological activity.

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