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IS THERE CORRELATION BETWEEN ANALGESIC POTENCY AND BIODEGRADATION OF ENKEPHALIN ANALOGS?

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Met-enkephalin and its Pro^5 analogs containing Gly or D amino acids at position 2 were subjected to digestion with aminopeptidase M, rat brain extracts and human sera. The enzyme resistance of these peptides was compared with their analgesic activity determined in tail flick test after central and intravenous administrations. Our data did not reveal an unambigous correlation between the analgesic potency and metabolic stability of the analogs. This suggest that analgesic activity of synthetic peptides should be due to factors other than enzyme resistance /e.g. receptor binding and transport properties/.

The discrepancy that enkephalins /1/, two brain peptides with sequence Tyr-Gly-Gly-Phe-Met and -Leu, possess remarkable morphine-like activities *in vitro* but cause extremely low and transient analgesia *in vivo* has been explained by rapid enzymic degradation of the peptides.Prompted by the suggestion that cleavage of the Tyr^1-Gly^2 bond is the initial deactivation step /2,3/ Pert *et al.* /4/ have synthesized /$D\text{-}Ala^2$,Met^5 /-enkephalinamide which has a stable N-terminus, i.e. Tyr^1-$D\text{-}Ala^2$, and causes profound and long-lasting analgesia when injected into rat brain. The assumption that the low analgesic potency of enkephalin is due to its high susceptibility to degradative enzymes has been further supported by recent studies on the degradation of opioid peptides by brain extracts /5,6/. Biodegradation was also considered when Pro^5 derivatives of enkephalin were prepared in our laboratory /7/. Namely the C-terminal Pro residue was expected to retard the

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action of carboxypeptidases. On the other hand, D amino acid residues were introduced with the aim of providing an extra side chain for enkephalin to bind to a possible additional site of the receptor/s/ /7/. Pro^5-enkephalins prepared possess analgesic activity even when injected intravenously whereas /D-Ala^2,Met^5/ -enkephalinamide applied in the same route fails to elicit analgesia /4/. Despite that peptides Tyr-D-Xxx-Gly-Phe-Pro are apparently of the same or similar resistance to enzymic degradation, their analgesic potency has been found to differ markedly. Therefore it seemed to be of interest to re-examine if there is a real correlation between analgesic potency and enzyme resistance of these peptides. In the present study Met-enkephalin and its Pro^5 analogs containing glycine or D amino acids, i.e. D-alanine, D-norleucine/Nle/, D-phenylalanine, D-methionine and D-ethionine /Eth/, at position 2 were subjected to hydrolysis with aminopeptidase M /APM/, with enzyme complex of rat brain extracts and of human serum, resp. The stability of these peptides was compared with their analgesic activity determined in the tail flick test in rats after intracerebroventricular /icv/ or intravenous /iv/ injection.

MATERIALS AND METHODS

Met-enkephalin and the analogs Tyr-Xxx-Gly-Phe-Pro-Q, wherein Xxx stands for Gly, D-Ala, D-Nle, D-Phe or D-Eth, and Q stands for OH or NH_2 were prepared as described previously /7-9/. For studies on the breakdown of enkephalins in rat brain extracts Marks' approach /10/ was used as reported by Patthy et al. /11/. Degradation by human serum was studied as follows: 0.6 ml of 0.01 M TRIS buffer /7.6/ containing 0.07 mg substrate /about 0.1 umole/ was added to 0.4 ml of fresh human serum and incubated at 37°C for 4 h, then a 0.5 ml aliquot of the deproteinized supernatant of each sample was subjected to amino acid analysis. APM digestions were performed in 1 ml 0.05 M TRIS buffer /pH 7.6/ containing 1 mg substrate /about 1.5 umole/ and 0.1 mg enzyme /Röhm Gmb/. The amount of amino acids released during digestions was determined in a JEOL /JLC-5AH/ automatic analyzer and expressed in mole-percentage of the substrate. The analgesic activity of peptides was assessed in rats using the tail flick test as described previously /12/.

RESULTS AND DISCUSSION

The action of APM on Met-enkephalin and its analogs was examined first since the involvement of aminopeptidases in deactivation of opioid peptides had been pointed out by several authors /2-6/. It has long been known that APM can hydrolyze any peptide bond except those formed by Pro or D amino acid residues.Our present findings /Table I/ partly supported the above notion. Namely Met^5- and Pro^5-enkephalin /I and II/ were hydrolyzed to the same extent with the exception that the Phe-Pro bond in II was not split off. In addition, D amino acid residues at position 2 /III-VIII/ retarded the action of APM, that is the release of Tyr^1. It also appeared, however, that liberation of Tyr is hindered by $D\text{-}Ala^2$ to a lesser extent than by any other D-amino acid residue having longer side chain. Moreover, D-Ala itself could be released in significan amount when III was digested for 3 or more hours. Finally, the C-terminal amide function of a pentapeptide, like IV, also seemed to affect the action of APM /cf. data of III and IV/. Data of the digestion with rat brain extracts and with human sera are given in Table II. Pro^5-enkephalin proved to be even more susceptible to brain and serum enzymes than the parent compound, i.e. Met-enkephalin. The action of aminopeptidase/s/ was averted by the D amino acid residues at position 2 in the brain extracts somewhat less efficiently than in sera. Interestingly enough, $D\text{-}Ala^2$ in III can completely block the release of Pro^5. Comparing the degradation of peptideamides IV-VIII minor differences can only be observed just like in the experiments with APM.

Albeit the enzymes examined in our experiments and those involved in biodegradation of opioid peptides are not identical, such studies may provide estimates for the susceptibility of

Table I

Degradation of enkephalins /Tyr-Xxx-Gly-Phe-Yyy-Q/ by aminopeptidase M

No. of com-pounds	changing residues		percent of amino acids released after 0,5 and 3 h, resp.									
	Xxx[2]	Yyy[5]-Q	Tyr		Xxx[2]		Gly		Phe		Yyy[5]	
			0.5	3 h	0.5	3 h	0.5	3 h	0.5	3 h	0.5	3 h
I	Gly	Met-OH	65	74	66	67	66	67	32	71	16	62
II	Gly	Pro-OH	67	72	58	64	58	64	0	0	0	0
III	D-Ala	Pro-OH*	37	63	+	6	+	9	0	0	0	0
IV	D-Ala	Pro-NH_2	27	55	0	+	0	+	0	0	0	0
V	D-Nle	Pro-NH_2	6	17	0	0	0	0	0	0	0	0
VI	D-Met	Pro-NH_2	5	14	0	0	0	0	0	0	0	0
VII	D-Eth	Pro-NH_2	5	10	0	0	0	0	0	0	0	0
VIII	D-Phe	Pro-NH_2	5	10	0	0	0	0	0	0	0	0

*after digestion for 18 h D-Ala was released in 28%, Tyr and Gly in 100 and 35%, resp.

Table II

Degradation of enkephalins /Tyr-Xxx-Gly-Phe-Yyy-Q/ by brain extract and serum

Number of compounds	changing residues Xxx[2]	Yyy[5]-Q	percent of amino acids released by brain extract after 0.5h /A/ and 2h /B/, resp., and by serum after 4h /C/: Tyr		Xxx[2]		Gly		Phe		Yyy[5]	
			A/ B	C	A/ B	C	A/ B	C	A/ B	C	A/ B	C
II	Gly	Pro-OH	77/88	99	50/56	71	50/56	71	62/67	78	90/100	100
I	Gly	Met-OH	60/60	91	25/42	39	25/42	39	51/56	76	54/74	41
III	D-Ala	Pro-OH	22/46	-	0/ 5	-	0/12	-	0/ 0	-	0/ 0	-
IV	D-Ala	Pro-NH_2	15/38	11	0/ 6	0	0/ 0	0	0/ 0	0	0/ 0	0
VII	D-Eth	Pro-NH_2	12/37	-	0/ 0	-	0/ 0	-	0/ 0	-	0/ 0	-
VI	D-Met	Pro-NH_2	8/22	0	0/ 0	0	0/ 0	0	0/ 0	0	0/ 0	0
V	D-Nle	Pro-NH_2	7/26	0	0/ 0	0	0/ 0	0	0/ 0	0	0/ 0	0
VIII	D-Phe	Pro-NH_2	0/15	0	0/ 0	0	0/ 0	0	0/ 0	0	0/ 0	0

peptides towards brain and serum proteinases. To characterize the enzyme resistance by numerical values percentage of intact peptide in hydrolysates was calculated. Such "stability" values together with the analgesic potencies are listed in Table III. Data indicate that the correlation between analgesic potency and biodegradation of the peptides strongly depends on the way of application. In case of central administration a "threshold resistance" of the peptides is required for the analgesic effect otherwise an analog, e.g. the extremely unstable Pro^5-enkephalin /II/, fails to cause any response. Beyond this threshold stability, however, the "stability" values do not give reliable information for the biological potencies. For explaining the order of analgesic potencies assessed after systemic administration, one must realize that the "stability" is of even less informatory value. This is clearly illustrated by the practically identical activity of II, III, and IV, three compounds of widely different susceptibility to proteinases. A comparison of the data for I and II strongly suggests that the lack of significant analgesic effect with Met-enkephalin applied intravenously cannot solely be due to its rapid degradation.

From the above data we may conclude that the extremely high biological potency of an enkephalin analog like /D-Met^2,Pro^5/-enkephalinamide is a result of the satisfaction of at least three or four different requirements, namely favourable transport properties, ability to cross the blood-brain barrier, enhanced or improved binding capacity and increased enzyme resistance. The significance of the latter one occurs to us to be overestimated in the literature. Notwithstanding that all the naturally occuring peptides undergo biodegradation, no difficulty has been encountered yet to demonstrate their in vivo biological effect,

Table III

"Stability" and analgesic potency of enkephalins /Tyr-Xxx-Gly-Phe-Yyy-Q/

Number of compounds	changing residues Xxx[2]	Yyy[5]-Q	"STABILITY" intact peptide in brain extract after 0.5/2h	in human serum after 4h	P O T E N C Y centrally 10^3 ED_{50} nM/animal	intravenously 10^3 ED_{50}/uM/kg
II	Gly	Pro-OH	10/ 0	0	⟨1	64
I	Gly	Met-OH	40/26	9	~1.5	0
III	D-Ala	Pro-OH	78/54	-	250	83
IV	D-Ala	Pro-NH_2	85/62	89	1538	89
VII	D-Eth	Pro-NH_2	88/63	-	5714	235
VI	D-Met	Pro-NH_2	92/78	100	24242	3125
V	D-Nle	Pro-NH_2	93/74	100	7407	222
VIII	D-Phe	Pro-NH_2	100/85	100	250	204

whilst the analgesic property of Met- and Leu-enkephalins - even if it is due to an extreme metabolic instability - cannot be of physiological significance. In this context it is interesting to note that the biological properties of /D-Met^2, Pro^5/-enkephalinamide are more reminiscent of those of β-endorphin than those of Met- and Leu-enkephalins /12, 13/. While Met- and Leu-enkephalins as well as their D amino acid containing analogs possess higher activity on mouse vas deferens than on guinea pig ileum, β-endorphin and /Met^2, Pro^5/-enkephalinamide are equipotent in the two preparations, in other words, the latters do not differentiate the same way the distinct opiate receptor populations of the two in vitro models as the formers do. Thus introduction of D-Met^2 and Pro-NH$_2^5$ into the structure and the addition of residues 6-31 to the Met-enkephalin moiety to form β-endorphin appear to exert analogous biological effects in this regard. A particular intramolecular interaction between the active site /residues 1-4/ and the C-terminal sequence portion of β-endorphin /14/ might also bring about all these favourable changes mentioned above.

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