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DERMORPHIN ANALOGS: RESISTANCE TO IN VITRO ENZYMATIC DEGRADATION IS NOT ALWAYS INCREASED BY ADDITIONAL D-AMINO ACID SUBSTITUTIONS

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SUMMARY: Dermorphin (H-Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2) and seven analogs were examined for their biostability towards rat brain homogenate; half-lives for the parent were 127 min and 48 min, respectively, with Gly^4-Tyr^5 cleavage confirmed by collection and identification of the N-terminal fragment. Surprisingly, several analogs with additional D-amino acid substitutions were cleaved more rapidly than the parent, suggesting the importance of remote secondary structural features for differential enzyme susceptibility.

Dermorphin (H-Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2), an opioid heptapeptide, was first isolated from the skin of frogs of the Phyllomedusa species (1). The presence of the unusual D-amino acid residue at the 2 position has been partially credited for dermorphin's very high and long lasting analgesic activity (2). Extensive structure-activity studies of dermorphin analogs have been reported in the literature (3-16).

The first studies of the enzymatic stability of dermorphin (17) confirmed the increased resistance of the molecule toward proteolytic enzymes. This enhanced stability was attributed both to the D-amino acid at position 2 as well as to the proline in position 6, both of which appear to protect dermorphin against exopeptidase action (18). In vivo experiments showed that dermorphin is degraded by the liver and kidney to the N-terminal tripeptide, and by brain enzymes to the N-terminal tetrapeptide (19). Further in vitro experiments demonstrated that both kidney and brain homogenates can inactivate dermorphin by cleaving the peptide bond between position Gly-4 and Tyr-5 (20,21). In contrast,

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in vitro experiments with plasma showed the very high stability of dermorphin in that environment (17).

We have previously shown that HPLC-based assays of peptides and pseudopeptides can be used to monitor and quantify both half-lives and cleavage patterns in biological fluids (23). Interestingly, the incorporation of amide bond surrogates was sometimes seen to increase the stability of remote scissile bonds. More recently this approach has been expanded to help design more stable and highly potent analogs of cholecystokinin octapeptides as anti-satiety agents (22). In this paper, we report the results of analogous experiments on dermorphin and a series of analogs with modifications at diverse locations throughout the molecule, and we contrast the degradation results with their biological activities.

MATERIALS AND METHODS

Chromatography. Peptides discussed in this study were previously synthesized in our laboratory (15,16). HPLC analyses were performed using an L-6200 Hitachi pump equipped with a solvent gradient system, a temperature controlled column oven, a Varian Model 9090 autosampler, and an LKB 2140 diode array detector, coupled to an IBM AT personal computer using Wavescan software (LKB, Bromma, Sweden, version 1.08). Degradation products were separated on an analytical Zorbax C_{18} column (4.6 x 250 mm) using a gradient made up of two solvents: A, 0.25 M ammonium acetate, pH = 6.5; B, methanol. The gradient used was of 5-20% methanol for 5 min, 20-40% methanol for 25 min, 40-50% methanol for 5 min. The elution program was initiated 1 min after sample injection, at a flow rate of 1 ml/min at 40°C. Degradation products after HPLC fractionation were collected and hydrolyzed in 6N hydrochloric acid for 24 h at 110°C. Amino acid analysis was performed using the PicoTag system (Waters Associates, Milford, MA).

Peptide Degradation. Degradation studies were performed using rat brain or dog duodenal homogenates. Rat brain homogenate was obtained from male Sprague-Dawley rats (350-450 g). Brain tissue was homogenized in 4 parts (w/v) saline at 0°C using a PCU-2 homogenizer (Kinematica GmbH, Luzern) using 3 x 10 sec bursts. Fractions of supernatant (0.5 ml for each peptide) obtained after centrifugation (3000 rpm for 15 min at 0°C) were used for assay. An average amount of 0.7-0.9 mg of peptide was dissolved in 2.0 ml of buffer (0.01 M aqueous ammonium acetate, pH = 7.4, containing 1.26 mM Z-Tyr-OH as internal standard), and 0.5 mL of brain homogenate was added. Incubations were carried out at 37°C. Aliquots of 200 µl were removed at variable time intervals up to 240 min, and the enzymatic reactions terminated by placing tubes into boiling water for 5 min. Samples were filtered through 0.46 µm nylon filters (Millipore, Bedford, MA) and then injected (100 µl) onto the reversed phase column. Degradation studies using dog duodenal homogenate were carried out as follows. The duodenum was surgically removed from a male German shepard, still on a respirator, after the removal of a kidney. The duodenal tissue was washed with saline solution and then homogenized in 9 parts (w/v) saline solution. A portion (200 µl) of this suspension was added to a glass vial containing a weighed sample (0.7-0.9 mg) of the appropriate peptide along with buffer and internal standard. The incubations, and further HPLC analysis were carried out as described above. Half-lives of dermorphin and its analogs were calculated as described previously (23).

RESULTS AND DISCUSSION

In the present study we have carried out in vitro analyses to assess the enzymatic stability of dermorphin and its analogs to rat brain and dog duodenal homogenates. Since it has previously been demonstrated that dermorphin is unusually more stable in blood serum (17,20), these tissues were deemed more suitable for rapidly comparing the relative stability of presumably more stabilized analogs.

The degradation half-lives for dermorphin and the other analogs tested are summarized in Table 1. Dermorphin showed a $t_{1/2}$ = 127 ±26.1 min (n=3) using rat brain homogenates, whereas inactivation using duodenal homogenate was faster ($t_{1/2}$ = 45.7 min). The same relative behavior was observed with the other dermorphin peptide analogs. All compounds modified in positions 1 to 5 showed lower stability than dermorphin. Generally, this finding correlates well with their lower in vitro activities (15,16), although this relationship is not necessarily predictable.

In contrast, [Sar^6]-dermorphin and [D-Ala^7]-dermorphin were found to be 6 and 2 times more stable, respectively, than the parent peptide, and showed in vitro GPI activities closer to the parent. Both of the latter two compounds are more active in vivo than is dermorphin (24). But [Tyr(Me)^5]-dermorphin showed a

TABLE 1. Half-lives of dermorphin and its analogs degraded by rat brain and dog duodenum homogenate

No.	Structure	Half-life (min) Brain (rat)	Duodenum (dog)	Rel. Activity GPI
1	H-Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2	127	45.7	1
2	Ac-Tyr^1----------NH_2	84.5	-	$3x10^{-5}$
3	H-D-Tyr^1----------NH_2	80.8	26.2	$3x10^{-4}$
4	H----------D-Phe^3----------NH_2	59.4	-	2.5x10-3
5	H----------Ala^5--------NH_2	59.2	54.3	$1.3x10^{-3}$
6	H----------Tyr(Me)^5----NH_2	112	-	4.5
7	H----------Sar^6----NH_2	860	-	0.38
8	H----------D-Ala^7-NH_2	257	-	0.55

TABLE 2. Degradation of dermorphin analogs by brain homogenate

No.	Structure	Peptide HPLC t_R/min	Main Degradation Peak HPLC t_R/min	amino acid analysis Tyr	Ala	Phe	Gly
1	Dermorphin	24.4	18.3	1.00	0.97	0.99	1.02
2	[Ac-Tyr^1]-Der	28.3	19.4	0.99	1.00	1.00	1.04
3	[D-Tyr^1]-Der	25.5	16.3	0.98	1.00	1.03	0.87
4	[D-Phe^3]-Der	23.2	16.2	0.99	1.20	1.02	1.00
5	[Ala^5]-Der	25.4	18.4	1.00	1.00	0.99	1.13
6	[Tyr(Me)^5]-Der	24.2	18.3	0.98	1.00	1.00	1.08
7	[Sar^6]-Der	23.3	18.3	n.d.	n.d.	n.d.	n.d.
8	[D-Ala^7]-Der	27.5	18.5	0.97	1.00	0.89	1.30

somewhat higher in vitro bioactivity, but was slightly less stable to enzymatic attack ($t_{1/2}$ = 112.1 min). Preliminary findings suggest that there is nevertheless an interesting positive correlation between the half-lives reported here and the in vivo analgesic activities of these compounds (24). However, much of this can be considered fortuitous since, for example, the D-Tyr^1 analog, when contrasted with other opioids, is not expected to retain high biological activity. On the other hand, the slightly increased rate of degradation observed both for this compound and for the D-Phe^3 analog is somewhat of a surprise, and reveals the importance of understanding the effects of remote modifications on enzymatic selectivity and substrate turnover rates.

Table 2 contains chromatographic data and amino acid analysis results of several metabolites collected after digestion by brain enzymes. On the basis of the amino acid analysis of the major fragment, all tested compounds gave results consistent with primary cleavage between positions 4 and 5 (Gly-Tyr). The predominant metabolite, the N-terminal tetrapeptide collected after assay appeared to be very stable against further degradation by brain homogenate. On the other hand the second metabolite, the C-terminal tripeptide, was not detected during assay. As reported earlier (20), this tripeptide appears to be further degraded very easily under the experimental conditions.

Our results are thus supportive of data published in the literature regarding both the relative stability and fragmentation patterns that can be expected for various dermorphin analogs. On the basis of the data from these studies, it would appear that analogs containing further stabilizing modifications in the interior portion, such as isosteric amide bond replacements, might increase their half lives and yet be consistent with retention or enhancement of biological potency. In this regard, it is interesting to note that Scalia et al. (20) have recently reported that a dermorphin analog with a retro amide ([NHCO]) replacement between Gly^4 and Tyr^5 blocked 4-5 cleavage, but did not substantially increase biological potency (25). Further efforts to improve both stability and potency are in progress in our laboratories.

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