DEHYDRO-ENKEPHALINS: RECEPTOR BINDING ACTIVITY OF UNSATURATED ANALOGS OF Leu^5-ENKEPHALIN

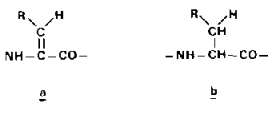
Yasuyuki SHIMOHIGASHI[+], Tommaso COSTA* and Charles H. STAMMER
Department of Chemistry, University of Georgia, Athens, GA 30602 and **National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20205, USA*

Received 27 July 1981; revision received 14 August 1981

1. Introduction

As part of our interest in the synthesis of dehydropeptides [2], we reported the preparation of a highly potent [D-Ala^2, ΔPhe^4, Met^5]-enkephalin amide [1], which had 5-times the activity of the corresponding saturated enkephalin in the stimulated guinea pig ileum assay and the mouse tail-flick test [3]. In general, the dehydroamino acid residue might enhance activity by virtue of an increased receptor-binding affinity, by its ability to react irreversibly [4] with a nucleophile on the receptor surface or by increasing resistance to enzymic degradation. A peptide messenger (hormone) most probably contains 'binding' and 'active' elements in its amino acid sequence [5]. The placement of a double bond into an amino acid residue in the 'binding' element could increase binding and elicit a stronger bio-response.

We have synthesized a series of unsaturated enkephalin analogs, dehydro-enkephalins (ΔEK), which contain ΔAla^2 [or 3], ΔPhe^4 or ΔLeu^5 residues (fig.1). Attempts to prepare ΔTyr^1-enkephalins have failed because of the hydrolytic instability of an N-terminal ΔTyr residue. This paper discusses the binding affinity and selectivity of these ΔEKs for 2 types of opiate receptors [6], and shows that incorporation of a dehydroamino acid residue is an effective way to obtain enkephalin analogs with full receptor activity which are resistant to enzymic hydrolysis without changing the selectivity.



amino acid		
unsaturated a	saturated b	R
ΔAla	Ala	H
ΔPhe	Phe	C_6H_5
ΔLeu	Leu	$CH(CH_3)_2$

Fig.1. The structures of unsaturated (a) and saturated (b) amino acid residues.

2. Materials and methods

2.1. *Peptide synthesis*

The peptides were synthesized by classical methods, and the dehydroamino acid moieties were prepared as in [7–9] and were confirmed by ^{1}N-NMR and UV difference spectroscopy [10]. All ΔPhe and ΔLeu peptides had the *z*-configuration [11]. Free ΔEKs were liberated by HF-anisole and purified by gel filtration (Bio-Gel P-2, aq. AcOH) followed by partition chromatography (Sephadex G-10, *n*-BuOH–AcOH–H_2O (4:1:5)). Homogeneity was determined by thin-layer chromatography, paper electrophoresis and amino acid analysis (before and after hydrogenation of the unsaturated moieties).

2.2. *Receptor binding assays*

Binding assays using [^{3}H][D-Ala^2, D-Leu^5]-enkephalin ([^{3}H]DADLE, 40 Ci/mmol) and [^{3}H]dihydromorphine ([^{3}H]DHM, 47.5 Ci/mmol) were performed as in [12]. Potencies expressed in ED_{50} have been estimated utilizing an RIA computer program as in [13].

3. Results and discussion

The substitutions of D-Ala^2 by ΔAla^2 (I), L-Phe^4 by ΔPhe^4 (III) and L-Leu^5 by ΔLeu^5 (IV) preserve

Paper 2 in the series Dehydro-enkephalins; paper 1 is [1]

[+] Present address: NIH, NICHD, Bethesda, MD 20205, USA

Published by Elsevier/North-Holland Biomedical Press
00145793/81/0000–0000/S02.75

Table 1
Binding activity of [D-Ala²]-dehydroenkephalins

ΔEK		[³H]DADLE			[³H]DHM			ED_{50} *vs* [³H]DHM
		ED_{50}[a] (±2 SEM)[b]		%Act.[c]	ED_{50} (±2 SEM)		%Act.	ED_{50} *vs* [³H]DADLE
[ΔAla², Leu⁵]	(I)	2.8	(2.2–3.5)	68	6.1	(6.4–6.9)	125	2.2
[D-Ala², ΔAla³, Leu⁵]	(II)	4.5	(3.5–5.7)	42	26	(21–32)	29	5.8
[D-Ala², ΔPhe⁴, Leu⁵]	(III)	1.9	(1.5–2.4)	100	6.4	(5.7–7.9)	119	3.4
[D-Ala², ΔLeu⁵]	(IV)	2.5	(1.9–3.2)	76	8.7	(6.9–10.1)	87	3.5
[ΔAla²]-desLeu⁵-OMe	(V)	180	(142–228)	1	76	(61–94)	10	0.4
[D-Ala², D-Leu⁵]	(ST_1)	1.9	(1.5–2.4)	100	7.6	(6.1–9.4)	100	4.0
[Leu⁵]	(ST_2)	2.4	(1.8–3.0)	79	15	(12–19)	51	6.2
β-Endorphin		2.3	(1.8–2.9)	83	2.8	(2.2–3.5)	270	1.2

[a] nM in the presence of bacitracin (100 μg/ml)
[b] Means and standard deviations from 3 or 4 independent assays were calculated assuming a log distribution of the variance [13], from the log value of the ED_{50}. The standard error of the mean (SEM) is based on a pooled estimate of the standard deviation, and hence on a higher value of degree of freedom
[c] Relative potency (%) against ST_1

almost full receptor activity (80–120%) as shown in table 1. These replacements result in a loss of chirality but a gain in rigidity and hydrophobicity. Since D-Phe⁴-enkephalins have no binding activity [14], position 4 apparently influences the conformation required for receptor binding. These results indicate that the ΔPhe⁴ moiety in III maintains this stereochemical requirement and sustains full binding activity (table 1). On the other hand, the considerable activity of [D-Ala², ΔLeu⁵]-enkephalin (IV) is presumably due to the insensitivity of the opiate receptors to chirality in position 5, because both L- and D-Leu⁵-enkephalins are fully active [14].

Unlike ΔPhe and ΔLeu, ΔAla, which has no substituents on β-carbon, must be the most electrophilic dehydroamino acid and, consequently, most reactive toward nucleophiles [2]. However, in these receptor binding assays, [ΔAla², Leu⁵]-enkephalin (I) showed almost the same affinities as the saturated analog (ST_1) and the ΔPhe⁴ and ΔLeu⁵ analogs. Replacement of Gly³ by different amino acids drastically reduces the opiate activities [14]. Surprisingly, [D-Ala², ΔAla³, Leu⁵]-enkephalin (II) maintains most of its activity both in [³H]DADLE (42%) and in [³H]DHM binding (29%).

These results suggest that 'dehydro-enkephalins' sustain a normal receptor interaction because the incorporation of dehydroamino acids confers the necessary structural elements: rigidity, hydrophobicity and conformation on the molecule. This change does not, however, produce the large enhancement of binding affinity which would be expected if covalent bond formation with the receptor surface were part of the binding mechanism.

The influence of a dehydroamino acid moiety on the selectivity of peptides for the two classes of opiate receptors [6] has been examined by using [³H]DADLE for peptide receptors (δ) and [³H]DHM for opiate receptors (μ) as tracers. The ratio of potencies in these two assays, ED_{50} using [³H]DHM/ED_{50} using [³H]DADLE suggests preferences for one of the two types of receptors (table 1). β-Endorphin has almost

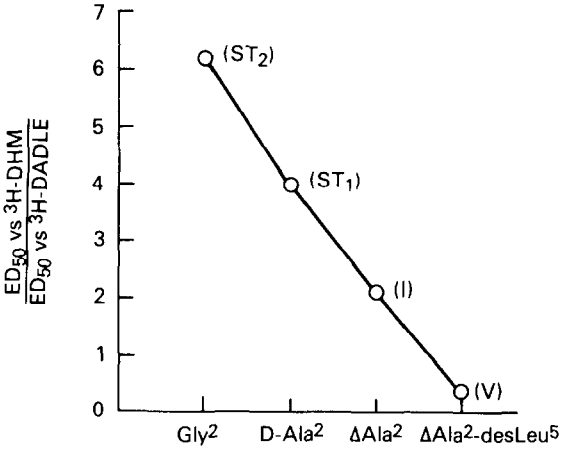


Fig.2. Potency ratio (ED_{50} *vs* [³H]DHM/ED_{50} *vs* [³H]-DADLE) for different substituents in position 2 of enkephalin: ratio = 1, equipotent; ratio >1, more potent for δ receptors; ratio <1, more potent for μ receptors.

the same affinity for both receptors, while enkephalins generally have a greater affinity for 'δ' receptors. Our ΔAla^2-tetrapeptide (V), which has weak activity, shows a higher selectivity for 'μ' receptors. This result is mainly due to the removal of the C-terminal residue [15]. As shown in fig.2, D-Ala^2-enkephalin prefers 'μ' receptors as compared with Gly^2-enkephalin (ST_2), and the replacement of D-Ala^2 by ΔAla^2 facilitates the shift of selectivity towards 'μ' receptors. These results indicate that an increase in hydrophobicity at position 2 causes a preference for the 'μ' receptors.

In contrast, ΔPhe^4-(III) and ΔLeu^5-(IV) enkephalins have a preference for 'δ' sites as do the parent saturated analog (ST_1). Increased hydrophobicity in positions 4 and 5 does not facilitate selective interactions with the receptors. It appears that the increase of hydrophobicity by α,β-dehydrogenation is most pronounced in case of ΔAla, and minimized in the more sterically congested ΔPhe and ΔLeu compounds.

The enzymatic degradation of enkephalins occurs most rapidly at Tyr^1–Gly^2 in rat brain homogenates [16] and in plasma [17], and also at Gly^3–Phe^4 by a dipeptidyl carboxypeptidase, enkephalinase [18]. Substitution of D-Ala^2 for Gly^2 gives a peptide with good agonist properties having a longer duration of action [16]. The chemical modifications (substitutions by D-amino acids, N-CH_3, amides and esters) of enkephalin, which provide resistance to these enzymic degradations, however, usually are associated with a loss of 'δ' *vs* 'μ' receptor selectivity [19]. Dehydropeptides have been shown to be resistant to enzymic hydrolysis [1,20,21]. Since analogs III and IV show the full receptor activity without changing selectivity, it appears that α,β-dehydrogenation, or incorporation of a dehydroamino acid, is an effective way to produce enkephalins resistant to enzymatic degradation with an unaltered full receptor affinity. To elucidate other effects of dehydroamino acid moieties additional biological assays are in progress.

Acknowledgements

We wish to thank Drs J. Travis and M. Morii, University of Georgia, for their amino acid analyses of our peptides. We are grateful to the National Institute for Drug Abuse for the financial support of grant DA02938.

References

[1] Paper, I., English, M. L. and Stammer, C. H. (1978) Biochem. Biophys. Res. Commun. 85, 780–782.
[2] Stammer, C. H. (1981) in: Chemistry and Biochemistry of Amino Acids, Peptides and Proteins (Weinstein, B. ed) vol. 6, in press.
[3] Chipkin, R. E., Stewart, J. M. and Stammer, C. H. (1979) Biochem. Biophys. Res. Commun. 87, 890–895.
[4] Breitholle, E. G. and Stammer, C. H. (1976) J. Org. Chem. 41, 1344–1349.
[5] Walter, R. (1977) Fed. Proc. FASEB 36, 1872–1878.
[6] Lord, J. A. H., Walerfield, A. A., Hughes, J. and Kosterlitz, H. W. (1977) Nature 267, 495–499.
[7] Photaki, I. (1963) J. Am. Chem. Soc. 85, 1123–1126.
[8] Bergmann, M., Schmidt, U. and Miekeley, A. (1930) Z. Physiol. Chem. 187, 264–276.
[9] Poisel, H. and Schmidt, U. (1976) Angew. Chem. Int. Ed. Engl. 15, 294–295.
[10] Shimohigashi, Y., Dunning, J. W. jr, Grim, M. D. and Stammer, C. H. (1981) J. Chem. Soc. Perkin Trans 2, in press.
[11] Srinivasan, A. and Olsen, R. (1976) Tetrahedron Lett. 891–894.
[12] Pert, C. B. and Snyder, S. H. (1974) Mol. Pharmacol. 10, 868–879.
[13] Fader, V. B. and Rodbard, D. (1975) Radioimmunoassay Data Processing, 3rd ed, vol. 1, Natl. Tech. Infor. Ser. Report no. PB246223.
[14] Beddell, C. R., Clark, R. B., Follenfant, R. L., Lowe, L. A., Ubatuba, F. B. and Miller, R. J. (1977) in: Biological Activity and Chemical Structure (Buisman, J. A. K. ed) pp. 177–193, Elsevier/North-Holland, Amsterdam, New York.
[15] Ronai, A. Z., Szekely, J. I., Berzetei, I., Miglecz, E. and Bajusz, S. (1979) Biochem. Biophys. Res. Commun. 91, 1239–1249.
[16] Pert, C. B., Pert, A., Chang, J. and Fong, B. (1976) Science 194, 330–332.
[17] Hambrook, J. M., Morgan, B. A., Rance, M. J. and Smith, C. F. C. (1976) Nature 262, 782–783.
[18] Malfroy, B., Swerts, J. P., Guyon, A., Roques, B. P. and Schwartz, J. C. (1978) Nature 276, 523–526.
[19] Chang, K.-J. and Cuatrecasas, P. (1979) J. Biol. Chem. 254, 2610–2618.
[20] English, M. L. and Stammer, C. H. (1978) Biochem. Biophys. Res. Commun. 83, 1464–1467.
[21] Grim, M. D., Chauhan, V., Shimohigashi, Y., Kolar, A. J. and Stammer, C. H. (1981) J. Org. Chem. 46, 2671–2673.