(D-Ala^2, N-Val^5) ENKEPHALINAMIDE AND (D-Met^2, N-Val)^5 ENKEPHALINAMIDE

Two potent agonists of opiate and enkephalin receptors

Y. AUDIGIER, H. MAZARGUIL and J. CROS

Laboratoire de Pharmacologie et de Toxicologie Fondamentales, CNRS, 205, route de Narbonne, 31078 Toulouse Cédex, France

Received 23 November 1979

1. Introduction

Since the discovery of the enkephalins [1], an extensive literature has been concerned with the relationship between the chemical modifications of the peptidic structure and the classical parameters of a morphinomimetic activity [2–6]. Among these synthetized analogs, it is noteworthy that (Pro)^5 analogs are the most active compounds, especially when the second amino acid is also replaced by either D-Ala or D-Met [3,4]; these last substitutions strongly decrease the metabolic deactivation induced by various peptidases [7,8]. However, the exceptional nature of proline, that is an amino acid in which the amine function is intracyclic, could possibly explain this high morphinomimetic activity. For these reasons, we were interested by the synthesis of an analog where the proline cycle is opened and for which the α-amino acid character is conserved: such an amino acid corresponds to norvaline (N-Val). Here we report the synthesis of two related pentapeptides, (D-Ala^2, N-Val^5)-enkephalinamide and (D-Met^2, N-Val^5)-enkephalinamide and their pharmacological activity on the opiate and the enkephalin receptors.

2. Materials and methods

2.1. *Chemical synthesis*

The enkephalin analogs were prepared as in [3]. The degree of purity was evaluated on thin-layer chromatography or by high-pressure liquid chromatography using a liquid chromatograph Waters ALC-204. The column (μ Bondapak C18/Corasil, inverse phase) was eluted with CH_3OH–H_2O (1:1). The enkephalin analogs were detected by their A_{254}.

2.2. *Binding measurements*

Binding studies with [^{3}H]etorphine were performed on a rat brain homogenate. Male Sprague-Dawley Rats (180–200 g) were killed by decapitation and the brain removed on ice. The cerebral tissue (*minus cerebellum*) was then homogenized with a Teflon–glass homogenizer (V = 1500 rev./min; 5 strokes) in 110 vol. 0.05 M Tris–HCl buffer (pH 7.4). The crude homogenate (800 μl) was incubated for 20 min at 37°C in the presence of 100 μl [^{3}H]etorphine (1 nM) and 100 μl unlabelled effector. Non-specific binding was determined with 1 μM Levorphanol. The reaction was stopped by the addition of 5 ml cold Tris-buffer and the resulting suspension was filtered under pressure on glass fiber filter Whatman GF/B. The filters were washed twice with 5 ml Tris-buffer, dried at 120°C for 15 min then counted in a Beckman scintillation counter using a scintillation mixture with 40% efficiency.

For (D-Ala)^2-Leu-[^{3}H]enkephalinamide binding, a washed homogenate from mouse brain was used. Male Swiss mice (18–20 g) were killed and the homogenate prepared as before with the following modification: the homogenate was centrifuged at 49 000 × g for 10 min and the resulting pellet was homogenized in 10 ml Tris-buffer. After centrifugation (49 000 × g; 10 min) the pellet was resuspended in sufficient Tris-buffer to obtain 1 mg protein/ml final conc. The binding assay was performed as previously described using 3.5 nM (D-Ala)^2-Leu^5-[^{3}H]enkephalinamide. Non-specific binding was measured in the

presence of 1 μM unlabelled ligand. Bound ligand was separated from free ligand and counted.

The IC_{50} values were calculated by linear regression from log-probit plots and each value represents the mean ± SEM obtained in 3–4 separate experiments.

2.3. *Biological activity on isolated organs*

Isolated ilea from guinea-pigs were placed in organ baths containing Krebs solution and then stimulated by field stimulation (0.1 Hz, 0.1 ms, maximal voltage). The longitudinal contractions were recorded isometrically.

The agonist potency (inhibitory effect) of morphine and enkephalin analogs were tested by cumulative dose–response curves as in [9].

Isolated vas deferentia from mice were prepared and used as above and in [10].

2.4. *Drugs*

Morphine was a gift Francopia, Paris; [^{3}H]etorphine (35.4 Ci/nmol) and (D-Ala)^2-tyrosyl-3,5-[^{3}H]enkephalin (5-L-leucine amide) (39.3 Ci/nmol) were purchased from the Radiochemical Center, Amersham.

3. Results and discussion

The data of table 1 and 2 clearly show that for the opiate receptor (N-Val)^5 analogs are always more affine or active than the (Pro)^5 analogs. It should be noted that the most important differences are observed when the (D-Ala)^2 derivatives are compared. Similar conclusions can be established for the enkephalin receptor from the data reported in table 1 and 2. For the (D-Ala)^2 analogs, the replacement of Pro by N-Val leads to a compound which is >10-times more active than the (Pro)^5 analog on both assays. The general conclusion that can be drawn from these findings is that the integrity of Pro cycle is not necessary for the in vitro activity. In fact (N-Val)^5 analogs are generally more active than the corresponding (Pro)^5 derivatives.

We have determined the influence of such amino acid replacement on the binding of analogs to the two types of receptors [11], that have been best characterized, i.e., opiate receptors (or μ receptors) and enkephalin receptors (or δ receptors). [^{3}H]Opiates have been reported to label opiate receptors which

Table 1
Affinity of morphine and enkephalin analogs for [^{3}H]etorphine sites and (D-Ala^2, Leu^5)-[^{3}H]enkephalinamide sites

Compound	[^{3}H]Etorphine IC_{50} (nm)	(D-Ala^2, Leu^5)-[^{3}H]Enkephalinamide IC_{50} (nM)
Morphine	170 ± 10	120 ± 19
(D-Met^2, Pro^5)-Enkephalinamide	82 ± 23	21 ± 1
(D-Met^2, N-Val^5)-Enkephalinamide	60 ± 22	18 ± 2
(D-Ala^2, Pro^5)-Enkephalinamide	340 ± 90	90 ± 14
(D-Ala^2, N-Val^5)-Enkephalinamide	58 ± 6	9 ± 2

Table 2
Agonist potency of morphine and enkephalin analogs on guinea-pig ileum and mouse vas deferens

Compound	Guinea-pig ileum IC_{50} (nM)	Mouse vas deferens IC_{50} (nM)
Morphine	80 ± 12	417 ± 75
(D-Met^2, Pro^5)-Enkephalinamide	14 ± 1	21 ± 2
(D-Met^2, N-Val^5)-Enkephalinamide	11 ± 1	17 ± 4
(D-Ala^2, Pro^5)-Enkephalinamide	50 ± 1	114 ± 35
(D-Ala^2, N-Val^5)-Enkephalinamide	25 ± 1	7 ± 2

are the majority of receptors present in guinea-pig ileum [11] and [^{3}H]peptides have been shown to characterize enkephalin receptors, mainly found in mouse vas deferens [11]. We have shown that there exists a strong correlation between the affinity for [^{3}H]etorphine binding site in rat brain and the inhibitory activity determined in guinea-pig ileum [12]. Similarly, the affinity for (D-Ala^2, Leu^5)-[^{3}H]enkephalinamide binding sites in mouse brain correlates with the depressant activity measured in mouse vas deferens [12]. We could therefore determine the influence of Pro and N-Val on the peptide affinity for the two types of receptors.

(D-Met)^2 compounds have been shown equiactive on opiate and enkephalin receptors [5–12] and replacement of Pro by N-Val does not modify this pharmacological profile nor affect the corresponding activity. However, very different results are obtained with (D-Ala)^2 analogs: whereas (D-Ala^2, Pro^5)-enkephalinamide is somewhat more active on opiate receptors than on enkephalin receptors, (D-Ala^2, N-Val^5)-enkephalinamide is considerably more active on both kind of receptors, especially on enkephalin receptors. Evidently replacing Pro with its chemical isomer N-Val alters the discrimination of enkephalins for the two types of opiate receptors.

In conclusion, (D-Met^2, N-Val^5)-enkephalinamide and particularly (D-Ala^2, N-Val^5)-enkephalinamide are two potent agonists of opiate and enkephalin receptors, more active than (D-Met^2, Pro^5)-enkephalinamide itself.

References

[1] Hughes, J., Smith, T. W., Kosterlitz, H. W., Fothergill, L. A., Morgan, B. A. and Morris, H. R. (1975) Nature 258, 577–579.
[2] Frederickson, R. C. A. (1977) Life Sci. 21, 23–42.
[3] Bajusz, S., Ronai, A. Z., Szekely, J. I., Dunai-Kovacs, Z. and Berzetei, I. (1977) FEBS Lett. 76, 91–92.
[4] Szekely, J. I., Ronai, A. Z., Dunai-Kovacs, Z., Miglecz, E., Berzetei, I., Bajusz, S. and Graf, L. (1977) Eur. J. Pharmacol. 43, 293–294.
[5] Shaw, J. S., Turnbull, M. J., Dutta, A. S., Gormley, J. J., Hayward, C. F. and Stacey, G. J. (1978) in: Characteristics and Function of Opioids, (Van Ree, J. M. and Terenius, L. eds) pp. 185–195, Elsevier/North-Holland, Amsterdam, New York.
[6] Miller, R. J., Chang, K. J., Cuatrecasas, P., Wilkinson, S., Lowe, L., Beddell, C. and Follenfant, R. (1978) in: Centrally Acting Peptides (Hughes, J. ed) pp. 195–213, MacMillans, London.
[7] Hambrook, J. M., Morgan, B. A., Rance, M. J. and Smith, F. C. (1976) Nature 263, 782–783.
[8] Pert, C. B., Pert, A., Chang, J. K. and Fong, B. T. W. (1976) Science 194, 330–332.
[9] Kosterlitz, H. W. and Watt, A. J. (1968) Brit. J. Pharmacol. 33, 266–276.
[10] Hughes, J., Kosterlitz, H. W. and Leslie, F. M. (1975) Brit. J. Pharmacol. 53, 371–381.
[11] Lord, J. A. H., Waterfield, A. A., Hughes, J. and Kosterlitz, H. W. (1977) Nature 267, 495–499.
[12] Audigier, Y., Mazarguil, H., Gout, R. and Cros, J. (1980) submitted.