SYNTHESIS AND OPIOID ACTIVITIES OF STEREOISOMERS AND OTHER D-AMINO ACID ANALOGS OF METHIONINE-ENKEPHALIN

D.H. Coy, A.J. Kastin, A.V. Schally, O. Morin, N.G. Caron, F. Labrie, J.M. Walker, R. Fertel, G.G. Berntson, and C.A. Sandman

Tulane University School of Medicine and Veterans Administration Hospital, New Orleans, Louisiana, 70112; Centre Hospitalier de l'Universite Laval, Quebec G1V 4G2, Canada; and Ohio State University, Columbus, Ohio, 43210.

Received October 1,1976

SUMMARY

A series of D-amino acid-substituted analogs of the opiate peptide, methionine^5-enkephalin, were synthesized by solid-phase methods and tested for their abilities to inhibit electrically-evoked contractions of mouse vasa deferentia and to compete with tritiated enkephalin for opiate receptors on particulate fractions isolated from homogenates of rat brain. [D-Ala^2]-enkephalin and [D-Ala^2]-enkephalin amide were found to be the most potent peptides in both assay systems, being about 1000% active in the vas deferens bioassay and 120% and 150% active, respectively, in the stereospecific binding test relative to methionine^5-enkephalin itself. In comparison, [D-Met^5]-, [D-Tyr^1]-, [D-Leu^2]-, [D-Phe^2]-, [D-Ala^3]-, and [D-Phe^4]-enkephalin had not more than 10% activity. The stabilization of the β-bend conformation of methionine^5-enkephalin by the substitution of D-alanine in position 2 of the peptide chain may contribute to the high activities of the [D-Ala^2]-analogs.

In the search for analogs of methionine^5-enkephalin (1), H-Tyr-Gly-Gly-Phe-Met-OH, with greater and more prolonged analgesic activity, it appeared to us that the substitution of D-amino acids into the peptide chain might offer a fruitful approach. This has certainly been the case with two of the hypothalamic peptide hormones, luteinizing hormone-releasing hormone (LH-RH) and somatostatin. Substitution of D-amino acids in position 6 of the LH-RH decapeptide, pGlu-His-Try-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2, results in greatly increased potency and duration of gonadotropin-releasing activity (2-4) and the replacement of the tryptophan residue in somatostatin with its D-isomer gives an analog with approximately 8 times the growth hormone-release inhibiting activity (5).

The situation with respect to the D-amino acid-6-analogs of LH-RH is particularly pertinent to the present study. The Tyr-Gly-Leu sequence of LH-RH has been postulated (6, 7) to exist in a β I-type bend conformation. The stabi-

lization of this conformation resulting from replacement of glycine by D-amino acids has been suggested (2, 6, 7) as a possible explanation, supported by minimum free-energy calculations, of the increased activity of this type of analog. A similar β-I bend conformation was proposed (8) to exist in the pentapeptide enkephalins, beginning, as with LH-RH, at tyrosine in position 1 with the second glycine residue in the i + 1 position. This model seems to have been fully confirmed by very recent NMR studies (9-11) on methionine^5-enkephalin.

Thus, replacement of glycine in position 2 of methionine^5-enkephalin by D-amino acids might be expected to yield analogs with increased activities. The replacement of glycine in position 3 by D-alanine and the remaining L-amino acid by their D-isomers was also undertaken in an attempt to determine other favorable conformational changes and perhaps to increase the resistance of the analogs to tissue peptidases.

METHODS

Peptide Synthesis:

The peptides described here were synthesized by solid phase methods with t-butyloxycarbonyl (Boc) protected amino acids. Those analogs with free carboxyl C-termini were assembled on a methionine-Merrifield resin [prepared from Boc-methionine and chloromethylated polystyrene resin by the method of Gisin (12)] using a cycle of events described previously (13). The hydroxyl group of tyrosine was protected with the 2-bromocarbobenzoxy group. At the completion of the solid-phase part of the synthesis, the final Boc-group on the tyrosine residue was removed by acidolysis before hydrogen fluoride cleavage to avoid alkylation of methionine (14). Peptide amides were similarly prepared on a benzhydrylamine resin (15).

Free peptides were liberated by treatment with hydrogen fluoride/anisole and were readily purified by gel filtration on Sephadex G-15 by elution with 0.2 M acetic acid (the more insoluble [D-Leu^2]- and [D-Phe^2]-analogs required 33% acetic acid as the eluant) followed by partition chromatography on Sephadex G-25 using the biphasic solvent system n-butanol: acetic acid: water (4:1:5). Homogeneity was demonstrated by thin layer chromatography in 4 solvent systems (Table I) and amino acid analysis of acid hydrolysates (Table II). Optical rotations are given in Table I.

Biological Assays:

Mouse vasa deferentia were prepared by the method of Hughes et al. (16). Tissues were suspended in 10 ml organ baths containing modified Krebs solution (16) at 35°C and oxygenated with 95% O_2-5% CO_2. Muscle contraction in response to rectilinear electrical pulses of 1.8 msec duration at 0.10 Hz at 100 volts produced at platinum electrodes set in the top and bottom of the bath were monitored by an isometric transducer in the absence and presence of various concentrations of enkephalin and enkephalin analogs, which were added in volumes of not more than 100 μl.

TABLE I

Physicochemical Properties of Enkephalin Analogs

Analog	$[\alpha]_D^\circ$ in 0.1 M AcOH at 26°	R_f (I)[a]	R_f (II)	R_f (III)	R_f (IV)
D-Tyr[1]	-41.1°(C, 1.01)	0.56	0.81	0.72	0.67
D-Ala[2]	40.4°(C, 0.69)	0.52	0.81	0.69	0.66
D-Leu[2]	40.4°(C, 1.05; 50% AcOH)	0.71	0.79	0.70	0.75
D-Phe[2]	-8.6°(C, 1.04; 50% AcOH)	0.65	0.80	0.66	0.78
D-Ala[3]	47.9°(C, 1.01)	0.53	0.81	0.69	0.64
D-Phe[4]	26.8°(C, 1.04)	0.50	0.71	0.63	0.62
D-Met[5]	45.4°(C, 0.83)	0.48	0.77	0.57	0.64
D-Ala[2]-amide	37.0°(C, 0.27)	0.56	0.82	0.56	0.62

[a]The following tlc solvent systems were used: R_f(I), n-BuOH: AcOH: H_2O (4:1:5, upper phase); R_f(II), EtoAc: pyridine: AcOH: H_2O (5:5:1:3); i-PrOH: 1 M AcOH (2:1); n-BuOH: AcOH: EtoAc: H_2O (1:1:1:1). Sample sizes of ca. 30 μg were spotted on silica gel plates and solvent was allowed to travel 10-15 cm. Spots were visualized with ninhydrin reagent followed by Cl-starch/KI reagent.

TABLE II

Amino Acid Analyses[a] of Enkephalin Analogs

Analog	Amino Acid Ratios
D-Tyr[1]	Tyr, 1.00; Gly, 1.95; Phe, 1.00; Met, 0.98
D-Ala[2]	Tyr, 1.01; Ala, 1.00; Gly, 0.97; Phe, 0.98; Met, 1.03
D-Leu[2]	Tyr, 0.99; Leu, 0.99; Gly, 1.00; Phe, 1.00; Met, 0.94
D-Phe[2]	Tyr, 0.99; Phe, 1.99; Gly, 1.00; Met, 1.00
D-Ala3	Tyr, 1.00; Gly, 1.00; Ala, 1.00; Phe, 1.00; Met, 1.02
D-Phe[4]	Tyr, 1.00; Gly, 1.97; Phe, 0.98; Met, 1.11
D-Met[5]	Tyr, 1.00; Gly, 2.04; Phe, 1.00; Met, 1.00
D-Ala[2]-amide	Tyr, 0.99; Ala, 1.04; Gly, 1.00; Phe, 1.00; Met, 0.95; NH_3, 0.95

[a]Amino acid analyses were performed on a Beckman 119 analyser equipped with a System AA computing integrator on samples which were hydrolyzed (110°, 18 h) in 4 M methanesulfonic acid containing 0.2% 3-(2-aminoethyl)indole.

The ability of methionine[5]-enkephalin and its analogs to bind to brain opiate receptors was determined by the method of Moran et al. (17). Approximately 100-250 μg of a particulate fraction [corresponding to fraction P2 isolated by Gray and Whittaker (18)], derived from whole rat brain homogenates from which the cerebella were removed, and 10 nmoles (80,000 cpm) of ^3H-

methionine^5-enkephalin (New England Nuclear, specific activity 15.15 Ci/mmole) were incubated at 0°- 4°for 70 minutes in a final volume of 0.5 Ml of buffer (25 mM Tris/HCl, pH 7.4 at 4°; 2 mM $MgCl_2$) in the presence or absence of increasing concentrations of enkephalin or its analogs. Stereospecific binding as measured by the displacement of radioactivity by 10^{-5} M of unlabeled methionine^5-enkephalin accounted for 60-70% of total binding. At the end of the incubation time, 2 ml of ice-cold buffer were added and the mixture was passed through glass fiber filters (Whatman GS-C) under reduced pressure and the residue washed three times with 5 ml of buffer. Radioactivity was determined after the addition of the residue to 10 ml of Aquasol. Assays were done in triplicate.

RESULTS AND DISCUSSION

Activities (Table III) of analogs in the mouse vas deferens bioassay were calculated from the molar concentrations which elicited a 50% reduction in strength of contraction (ED_{50}) and are compared to the values obtained with methionine^5-enkephalin itself. The molar concentrations of methionine^5-enkephalin and its analogs necessary to displace 50% of specifically bound ^{3}H-enkephalin from the membrane preparations were calculated from dose-response curves. Activities are given in Table IV.

Most dramatic was the high activity of [D-Ala^2]-enkephalin in both test systems employed. It appeared to have superior affinity for opiate receptors, but was even more active in the vas deferens bioassay. In a separate study, the analgesia produced by this analog after direct injection into the brain was found to be considerably greater than that elicited by methionine^5-enkephalin itself, being both more intense and persistent (Walker, Berntson, Sandman, Coy, Schally, and Kastin, unpublished observations). The [D-Ala^2]-analog also was more active than methionine^5-enkephalin in a dopa-potentiation assay (19). Thus, the possibility certainly exists that the [D-Ala^2]-modification does indeed stabilize the β-I bend conformation of enkephalin as it does with LH-RH. However, the increased activity of the peptide in vivo suggests that increased resistance to enzymatic degradation must also play an important role in producing such high activity. In comparison, [L-Ala^2]-enkephalin has been reported (20) to have only 3% of the activity of methionine^5-enkephalin in a stereospecific binding assay.

Unexpectedly, the [D-Leu^2]- and [D-Phe^2]-peptides were much less potent,

TABLE III

Activities of Methionine^5-Enkephalin Analogs in the Mouse Vas Deferens Bioassay

Peptide	ED_{50} (M)	% Activity
Met^5-enkephalin	1.1×10^{-8}	100
D-Tyr^1	$> 1 \times 10^{-5}$	< 0.1
D-Ala^2	1×10^{-9}	1000
D-Ala^3	$> 1 \times 10^{-5}$	< 0.1
D-Phe^4	$> 1 \times 10^{-5}$	< 0.1
D-Met^5	5×10^{-6}	0.2
D-Ala^2-amide	1×10^{-9}	1000

TABLE IV

Molar Affinities of Methionine^5-Enkephalin Analogs for Opiate Receptor Preparations

Peptide	% Receptor Affinity Relative to Met-Enkephalin
Met^5-enkephalin	100
D-Tyr^1	< 1
D-Ala^2	120
D-Leu^2	< 1
D-Phe^2	< 1
D-Ala^3	10
D-Phe^4	< 1
D-Met^5	10
D-Ala^2-amide	150

having less than 1% activity in displacing ^{3}H-enkephalin from opiate receptors. These results are contrary to those obtained (4) with LH-RH where increased size of the side-chain of the D-amino acid replacing glycine-6 resulted in large increases in gonadotropin-releasing activities. Apparently, bulky side-chains in position 2 of methionine^5-enkephalin have adverse effects, presumably

steric in character and possibly involving the adjacent tyrosine residue which is believed to be the active center of the enkephalin peptides.

[D-Ala^3]-enkephalin had less than 0.1% activity in the vas deferens bioassay and about 10% opiate binding activity, similar to the 6% activity reported (20) for [L-Ala^3]-enkephalin in an opiate binding assay. [D-Tyr^1]- and [D-Phe^4]-enkephalin were inactive at the doses tested; however, the [D-Met^5]-peptide possessed about 0.2 - 10% activity in both systems.

It has been reported (21) that methionine^5-enkephalin amide is approximately twice as effective as methionine^5-enkephalin in an opiate receptor-binding assay. This result is consistent with our finding that [D-Ala^2]-enkephalin amide is more potent than the [D-Ala^2]-analog in this type of assay and about equally active in the vas deferens bioassay. Possibly this peptide amide might be even more effective than [D-Ala^2]-enkephalin for induction of analgesia since it has a blocked C-terminus resistant to carboxypeptidase action.

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. Monahan, M.W., Amoss, M.S., Anderson, H.A., and Vale, W. (1973) Biochemistry, 12, 4616-4620.
3. Vilchez-Martinez, J.A., Coy, D.H., Arimura, A., Coy, E.J., Hirotsu, Y., and Schally, A.V. (1974) Biochem. Biophys. Res. Commun., 59, 1226-1232.
4. Coy, D.H., Vilchez-Martinez, J.A., Coy, E.J., and Schally, A.V. (1976), J. Med. Chem., 19, 423-425.
5. Rivier, J., Brown, M., and Vale, W. (1975) Biochem. Biophys. Res. Commun., 65, 746-751.
6. Momany, F.A. (1976) J. Amer. Chem. Soc., 98, 2990-2996.
7. Momany, F.A. (1976) J. Amer. Chem. Soc., 98, 2996-3000.
8. Bradbury, A.F., Smyth, D.G., and Snell, C.R. (1976) Nature, 260, 165-166.
9. Garbay-Jaureguiberry, C., Roques, B.P., Oberlin, R., Anteunis, M., and Lala, A.K. (1976) Biochem. Biophys. Res. Commun., 71, 558-565.
10. Roques, B.P., Garbay-Jaureguiberry, C., Oberlin, R., Anteunis, M., and Lala, A.K. (1976), Nature, 262, 778-779.
11. Jones, C.R., Gibbons, W.A., and Garsky, V. (1976) Nature, 262, 779-782.
12. Gisin, B.F. (1973) Helv. Chim. Acta, 56, 1476-1482.
13. Coy, D.H., Coy, E.J., Redding, T.W., and Schally, A.V. (1973) Biochem. Biophys. Res. Commun., 54, 1267-1270.
14. Noble, R.L., Yamashiro, D., and Li, C.H. (1976) J. Amer. Chem. Soc., 98, 2324-2328.
15. Pietta, P.G. and Marshall, G.R. (1970) Chem. Commun., 650.
16. Henderson, G., Hughes, J., and Kosterlitz, H.W. (1972) Brit. J. Pharm., 46, 1764-1766.

17. Moran, O., Caron, N.G., Delean, A., and Labrie, F. (1976) J. Molec. Endocrin., in press.
18. Gray, E.G. and Whittaker, V.P. (1962) J. Anat., 96, 79-96.
19. Plotnikoff, N.P., Kastin, A.J., Coy, D.H., Christensen, C.W., Schally, A.V., and Spirtes, M.A. (1976) Life Sci., 19, in press.
20. Terenius, L., Wahlstrom, A., Lindeberg, G., Karlsson, S., and Ragnarsson, U. (1976) Biochem. Biophys. Res. Commun., 71, 175-179.
21. Chang, J.K., Fong, B.T.W., Pert, A., and Pert, C.B. (1976) Life Sci., 18, 1473-1482.