Vol. 106, No. 2, 1982 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 31, 1982 Pages 506-512

[D-PEN^2, L-CYS^5]ENKEPHALINAMIDE AND [D-PEN^2, D-CYS^5]ENKEPHALINAMIDE, CONFORMATIONALLY CONSTRAINED CYCLIC ENKEPHALINAMIDE ANALOGS WITH DELTA RECEPTOR SPECIFICITY

Henry I. Mosberg*, Robin Hurst*, Victor J. Hruby*, James J. Galligan**, Thomas F. Burks**, Kelvin Gee**, and Henry I. Yamamura**

Departments of Chemistry* and Pharmacology**, University of Arizona, Tucson, AZ 85721

Received April 22,1982

The conformationally constrained cyclic enkephalin analogs, [2-D-penicillamine, 5-L-cysteine]- and [2-D-penicillamine, 5-D-cysteine]enkephalinamide were synthesized and their biological activities investigated. Both analogs effectively induced thermal analgesia as measured by the in vivo hot plate test. Both analogs were effective in inhibiting muscle contractions in the guinea pig ileum and mouse vas deferens assay systems and were shown to displace both [^{3}H]naloxone and [^{3}H][D-Ala^2, D-Leu^5]enkephalin from rat brain receptor preparations. The analogs exhibited a significant preference for δ-receptors over μ-receptors, an unusual finding for enkephalinamide derivatives. In addition the 5-L-cysteine containing analog was more potent than the 5-D-cysteine analog in all the in vitro assays with the exception of the guinea pig ileum system. These uncommon results are attributed to the conformational constraints imposed by the cyclization via a disulfide and by the rigidizing effect of the penicillamine.

INTRODUCTION

Since their isolation and sequence determination (1) the enkephalins have been the subject of intense structure-activity and conformational analyses. While the former studies have been useful in determining functional group requirements for enkephalin bioactivity, conformational studies have yielded many conflicting models of enkephalin solution and receptor-bound geometries (2-7). This lack of agreement is due at least in part to the inherent flexibility of small, linear peptides in (especially aqueous) solution. Further, the conformation of a flexible peptide is strongly influenced by its environment and the perturbing effect of a biological receptor may well favor a binding conformation of the peptide different from a highly populated solution conformation.

Abbreviations used: A_2bu, α,γ-diaminobutyric acid; Pen, half-penicillamine; GPI, guinea pig ileum; MVD, mouse vas deferens.

0006-291X/82/100506-07$01.00/0

These problems can be ameliorated to a large extent by the design of conformationally constrained peptide analogs. Such analogs have a greatly reduced number of possible solution conformations and, if sufficiently rigid, must maintain a similar conformation when bound to the receptor. In this case spectroscopic techniques can yield a reasonable solution conformation and the inference of receptor bound geometry is realistic.

Conformational restrictions of enkephalin analogs have generally been of two types. Local constraints such as α or N-methylation of the peptide backbone (8-10) yield analogs with limited conformational freedom but affect only the methylated residue (in the case of α methylation) or the methylated and preceeding residues (in the case of N-methylation) (11). Cyclic analogs such as H-Tyr-cyclo(-N^{α}-D-A_2bu-Gly-Phe-Leu) (12) and H-Tyr-D-Cys-Gly-Phe-L (or D)-Cys-NH_2 (13) have more far reaching effects since in these cases many of the conformations allowed for a linear analog can be discarded. Nonetheless, cyclization is an insufficient criterion for conformational rigidity. Thus, for example, the disulfide containing ring portion of oxytocin retains considerable flexibility in aqueous solution (14, 15).

Increased rigidity can be conferred on a disulfide-containing peptide if half-cystine is replaced by half-penicillamine (ββ-dimethyl half-cystine) due to the effect of gem dimethyl substitution in medium sized rings (16, 17). For example l-half-penicillamine oxytocin antagonist analogs have been found to have greatly reduced flexibility compared with oxytocin (17-19). The present paper describes the syntheses and biological activities of two half-penicillamine containing, cyclic enkephalin analogs, [D-Pen^2, D-Cys^5]-enkephalinamide and [D-Pen^2, L-Cys^5]enkephalinamide (Figure 1), designed to incorporate this conformational constraint for the purpose of elucidating the 3-dimensional features necessary for receptor binding and transduction.

MATERIALS AND METHODS

Peptides. The enkephalin analogs were synthesized by solid phase methods similar to those previously reported (20, 21), using polystyrene resin (1% cross-linked with divinylbenzene and chloromethylated to an extent of 1.06 mmol/g resin; Lab Systems, Inc., San Mateo, CA). N^{α}-Boc protected amino acids were used throughout and benzyl protection was utilized for the penicillamine

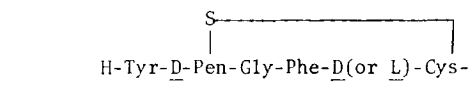

Figure 1. [D-Pen^2, D-Cys^5]- and [D-Pen^2, L-Cys^5]enkephalinamides.

and cysteine sulfurs. Tyrosine was used without side chain protection. The protected peptide was cleaved from the resin by treatment with a solution of freshly distilled anhydrous methanol saturated at -5° with anhydrous ammonia. The mixture was stirred at room temperature for 4 days and the solvents removed in vacuo. The peptide was extracted with dimethylformamide (DMF) at 50° with stirring for 18 hrs. The resin was filtered and extracted again with DMF for 3 hrs at 75°. The DMF fractions were pooled, the volume reduced to 5-10 ml and the peptide precipitated by slow addition of H_2O.

The protected pentapeptide was dissolved in anhydrous ammonia and treated with sodium to remove the benzyl protecting groups of the sulfur containing amino acids. The ammonia was removed under nitrogen and by lyophilization and the resulting powder dissolved in deaerated 0.1% aqueous acetic acid to a concentration of approximately 0.2 mM. The pH of the solution was adjusted to 8.0 with 3 N NH_4OH and the peptide was oxidized with 0.01 N $K_3Fe(CN)_6$ for 40 min. to form the disulfide. The pH was adjusted to 5 with 20% aqueous HOAc and ion exchange resin (Rexyn 203 Cl^- cycle) was added to remove ferro- and excess ferricyanide. After 20 min the resin was filtered, washed with 20% HOAc and the combined aqueous solutions were evaporated in vacuo to about 200 ml followed by lyophilization.

The peptides were purified by gel chromatography on Sephadex G-15 using 30% aqueous HOAc as eluent solvent followed by partition chromatography on Sephadex G-25 block polymerizate using the solvent systems butanol: HOAc: H_2O (4:1:5). Final purification was effected by gel chromatography on Sephadex G-25 block polymerizate using 20% HOAc as eluent solvent. Homogeneity of the peptides was established by thin layer chromatography (TLC) in the following systems, each of which yielded a single uniform spot for each peptide: i) chloroform/methanol/acetic acid (CMA) (65:35:4); ii) 1-butanol/acetic acid/water (BAW, upper phase only) (4:1:5); iii) 1-butanol/water (3.5% acetic acid, 1.5% pyridine) (BW, upper phase only) (1:1); and iv) 1-butanol/pyridine/acetic acid/water (BPAW) (15:10:3:12).

[D-Pen^2, D-Cys^5]enkephalinamide: TLC Rf: 0.27 (CMA), 0.53 (BAW), 0.36 (BW), 0.86 (BPAW). Amino acid analysis: Tyr 1.02, half-Cys + half-Pen 1.68, Gly 0.95, Phe 1.03.

[D-Pen^2, L-Cys^5]enkephalinamide: TLC Rf: 0.27 (CMA), 0.53 (BAW), 0.35 (BW), 0.85 (BPAW). Amino acid analysis: Tyr 1.02, half-Cys + half-Pen 1.79, Gly 0.96, Phe 1.02.

In Vivo Bioassays. Female Sprague Dawley rats (250-300g) were anesthetized with ketamine HCl (100 mg/kg ip.) and polyethylene cannulas (PE. 10, Clay Adams, Parsipanny, New York) were inserted in the right lateral cerebral ventricle (2 mm lateral, 2 mm posterior to bregma). A small stainless steel anchoring screw was threaded into the skull and the cannula was secured in place with a small mound of dental acrylic. The animals were housed individually and were allowed to recover for 72 hours. Drugs were dissolved in saline and intracerebral injections were administered in a volume of 5 µl. Analgesia was assessed by measuring the latency to rear paw lick when the animal was placed on a 55.5°C hot plate 10, 20, 40, and 120 minutes following peptide treatment. A fifty second cut-off was used and animals not responding within this time period were considered analgesic. Some animals were pretreated with naloxone (2 mg/kg ip.) ten minutes prior to peptide treatment.

In Vitro Bioassays. The guinea pig ileum longitudinal muscle-myenteric plexus preparation was prepared after the method of Kosterlitz et al. (22). Agonists were added to the tissue bath and remained in contact with the tissue for a maximum of three minutes. Concentrations were randomized and were added at intervals of fifteen minutes, during which the tissue was washed several times. This procedure was used to avoid the development of acute tolerance.

The mouse vas deferens was prepared after the method of Hughes et al. (23). One pair of vasa deferentia was used in each experiment. The buffer did not contain Mg^{2+} ions and a similar dose cycle was used as in the GPI.

Inhibition of Opiate Receptor Binding. Brains from male Sprague-Dawley rats were rapidly removed following sacrifice. Whole brains with cerebellum removed were homogenized in 100 volumes of Tris-HCl buffer (pH 7.4 @ 25°C). Tissue homogenates were washed twice in the same buffer by centrifugation at 48,000 x g for 10 min. Pellets were resuspended in the same buffer. Inhibition of opiate receptor binding was determined by incubating 100 µl of brain homogenate (5% original wet w/v) with 1 nM of [^{3}H][D-Ala^2, D-Leu^5]enkephalin (31.3 Ci/mmol, New England Nuclear Inc.) and varying concentrations of either [D-Pen^2, D-Cys^5]enkephalinamide, [D-Pen^2, L-Cys^5]enkephalinamide, [Leu^5]-enkephalin, [Met^5]enkephalin, or morphine hydrochloride at 25°C. Total incubation volume was 2 ml. After 40 min, the mixture was rapidly filtered through Whatman GF/B glass fiber filters and washed 3X with 5 ml of ice cold buffer. Minor modifications of this procedure were followed when [^{3}H]naloxone (37.7 Ci/mmol, New England Nuclear Inc.) was used to label opiate receptors. One hundred microliters of brain homogenate (2% original wet w/v) were incubated with 1.3 nM of [^{3}H]naloxone for 2 hours at 4°C. Filter bound radioactivity was quantitated by liquid scintillation spectrophotometry with an efficiency of 46%. Non-specific binding was defined as [^{3}H][D-Ala^2, D-Leu^5]-enkephalin or [^{3}H]naloxone bound in the presence of 1 µM leucine-enkephalin or 1 µM naltrexone, respectively.

RESULTS AND DISCUSSION

[D-Pen^2, L-Cys^5]-, and [D-Pen^2, D-Cys^5]enkephalinamide were both effective (3.3 µg, icv.) in increasing the hot plate latencies to the cut-off in 3 of 4 animals treated with each peptide. This analgesic effect was abolished by pretreatment with naloxone indicating a similar mechanism of action as other opiate agonists. The analgesic effect in animals treated with either peptide alone lasted 20-30 minutes. Higher doses of the peptide (10 µg) did not increase the number of animals reaching the cut-off but did prolong the duration of the analgesia.

Both D-Pen^2 enkephalinamide analogs were effective at inhibiting electrically evoked muscle contractions in both the GPI and MVD assay systems (Table I). These responses were sensitive to naloxone antagonism (10 and 200 nM). The D-Pen^2 analogs exhibit an unusual activity profile for enkephalinamide derivatives in that they are considerably more potent in the MVD assay than in the GPI assay. Since it is generally agreed that the MVD prepara-

Table 1. Guinea Pig Ileum and Mouse Vas Deferens Assays

Agonist	IC_{50} (nM) GPI	MVD	GPI/MVD
Normorphine	91 ± 19	540 ± 113	.17
[D-Ala^2, D-Leu^5]enkephalin	24.3 ± 5.3	0.27 ± 0.06	90
[D-Ala^2, Met^5]enkephalinamide	2.20 ± 0.4	3.75 ± 0.04	0.59
[D-Pen^2, D-Cys^5]enkephalinamide	117 ± 21.4	16.8 ± 3.1	6.9
[D-Pen^2, L-Cys^5]enkephalinamide	118 ± 18.6	3.6 ± 0.67	32.4

IC_{50} values and standard errors of the mean are given in nanomolar concentrations for opiate agonists in the GPI and MVD preparations. Reported values were obtained in 3-5 preparations for each compound.

tion contains largely δ receptors and the GPI preparation largely μ receptors (24), these results indicate a marked preference of these D-Pen^2 enkephalinamide analogs for δ receptors.

This increased specificity for δ receptors is also reflected in the results of the rat-brain receptor binding assays (Table 2). Both D-Pen^2 analogs are considerably more effective at displacing [^{3}H][D-Ala^2, D-Leu^5]-enkephalin, a ligand highly specific for δ receptors, than at displacing [^{3}H]-

Table 2. Binding Affinities for Rat Brain Receptor Preparations

Analog	IC_{50} (nM)[a] [^{3}H]naloxone	[^{3}H][D-Ala^2, D-Leu^5]-enkephalin	$\frac{IC_{50}^{\mu}}{IC_{50}^{\delta}}$[b]
[D-Pen^2, D-Cys^5]enkephalinamide	162.4 ± 34.8	7.20 ± 1.8	22.6
[D-Pen^2, L-Cys^5]enkephalinamide	73.4 ± 14.9	3.35 ± 0.15	21.9
Morphine·HCl	23.3 ± 2.4	27.2 ± 1.2	0.9
[Leu^5]enkephalin	35.0 ± 1.9	1.15 ± 0.15	30.4
[Met^5]enkephalin	27.0 ± 6.4	1.75 ± 0.05	15.4

[a] IC_{50} values and standard errors of the mean in nanomolar concentrations for displacing appropriate [^{3}H] ligand. [b] Ratio of IC_{50} values for displacement of [^{3}H]naloxone (μ receptor ligand) to displacement of [^{3}H][D-Ala^2, D-Leu^5]-enkephalin (δ receptor ligand). Reported values are the mean of two preparations each assayed in triplicate.

naloxone, a ligand specific for μ receptors. This finding can be contrasted with the reported results for the analogous [D-Cys^2, D-Cys^5]- and [D-Cys^2, L-Cys^5]enkephalinamides (13) both of which exhibit a moderate preference for μ receptors. These results suggest that the combined conformational constraints imposed by cyclization via the disulfide and by the penicillamine gem dimethyl groups lead to a conformation more favorable for binding to δ receptors.

While the two analogs are equipotent in the GPI assay, the L-Cys^5 derivative is ca. 2-5 times more potent than the D-Cys^5 derivative in the MVD and rat-brain receptor assays. This is counter to the generally observed trend in which the D enantiomer in position 5 yields a more potent analog than the corresponding L enantiomer. This again can be attributed to the greater rigidity of the present analogs. The L-Cys^5 containing diastereoisomer is apparently more able to attain the necessary 3-dimensional structures required for interaction with the δ and perhaps the μ receptors. Examination of the conformations of these compounds is currently in progress.

ACKNOWLEDGEMENTS

This research was supported by grants from the U. S. Public Health Service AM-17420 (VJH), DA 02163 (TFB), NS 15420 (TFB), MH 27257 (HIY), MH 30626 (HIY), a Research Scientist Development Award MH 00095 (HIY), by the National Science Foundation (VJH), and by a PMAF Predoctoral Fellowship (JJG).

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. Roques, B. P., Garbay-Jaureguiberry, C., Oberlin, R., Anteunis, M., and Lala, A. K. (1976) Nature 262, 778-779.
3. Jones, C. R., Gibbons, W. A., and Garsky, V. (1976) Nature 262, 779-782.
4. Bleich, H. E., Cutnell, J. D., Day, A. R., Freer, R. J., Glasel, J. A., and McKelvy, J. F. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 2589-2593.
5. Zetta, L., Cabassi, F. Tomatis, R., and Guarneri, M. (1979) Eur. J. Biochem. 95, 367-376.
6. Higashijima, T., Kobayashi, J., Nagai, U., and Miyazawa, T. (1979) Eur. J. Biochem. 97, 43-57.
7. Spirtes, M. A., Schwartz, R. W., Mattice, W. L., and Coy, D. H. (1978) Biochem. Biophys. Res. Commun. 81, 602-609.
8. Dutta, A. S., Gormley, J. J., Hayward, C. F., Morley, J. S., Shaw, J. S., Stacy, G. J., and Turnbull, M. T. (1977) Acta Pharm. Suec., Suppl. 14, 14-15.
9. Pless, J., Bauer, W., Cardinaux, F., Closse, A., Hauser, D., Huguenin, R., Roemer, D., Buescher, H. H., and Hill, R. C. (1979) Helv. Chim. Acta 62, 398-411.
10. Marshall, G. R. and Gorin, F. A. (1977) in Peptides, Goodman, M. and Meienhofer, J., eds., John Wiley and Sons, New York, 84-87.

11. Marshall, G. R. and Bosshard, H. E. (1972) Circ. Res. 30, 31, Suppl. 2, II-143-II-150.
12. DiMaio, J. and Schiller, P. W. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 7162-7166.
13. Schiller, P. W., Eggiman, B., DiMaio, J., Lemieux, C., and Nguyen, T. M.-D. (1981) Biochem. Biophys. Res. Commun. 101, 337-343.
14. Brewster, A. I. R. and Hruby, V. J. (1973) Proc. Natl. Acad. Sci. U.S.A. 70, 3806-3809.
15. Deslauriers, R., Smith, I. C. P., and Walter, R. (1974) J. Amer. Chem. Soc. 96, 2289-2291.
16. Eliel, E. L., Allinger, N. L., Angyal, S. J., and Morrison, G. A. (1965) Conformational Analysis, pp. 191-193, Interscience Publishers, New York.
17. Meraldi, J. P., Hruby, V. J., and Brewster, A. I. R. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 1373-1377.
18. Mosberg, H. I., Hruby, V. J., and Meraldi, J. P. (1981) Biochemistry 20, 2822-2828.
19. Mosberg, H. I. and Hruby, V. J. (1981) in Peptides: Synthesis, Structure, Function, Rich, D. H. and Gross, E., eds., Pierce Chemical Co., 375-378.
20. Hruby, V. J., Deb, K. K., Yamamoto, D. M., Hadley, M. E., and Chan, W. Y. (1979) J. Med. Chem. 22, 7-12.
21. Hruby, V. J., Mosberg, H. I., Hadley, M. E., Chan, W. Y., and Powell, A. M. (1980) Int. J. Pept. Prot. Res. 16, 372-381.
22. Kosterlitz, H. W., Lydon, R. J., and Watt, A. J. (1970) Br. J. Pharmacol. 39, 398-413.
23. Hughes, J., Kosterlitz, H. W., and Leslie, F. M. (1975) Br. J. Pharmacol. 53, 371-381.
24. Lord, J. A. H., Waterfield, A. A., Hughes, J., and Kosterlitz, H. W. (1977) Nature 267, 495-499.