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CYCLIC ENKEPHALIN ANALOGS CONTAINING A CYSTINE BRIDGE

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SUMMARY

Two conformationally constrained enkephalin analogs were synthesized by substitution of cysteines in positions 2 and 5 and oxidative disulfide bond formation. In the guinea pig ileum assay the obtained cyclic analogs, [D-Cys^2-L-Cys^5]enkephalinamide and [D-Cys^2-D-Cys^5]enkephalinamide, showed potency ratios of 37.9 ± 0.8 and 73.3 ± 0.9, respectively, relative to [Met^5]enkephalin. The extremely high potency of the analogs was shown to be a consequence of the conformational restrictions introduced by cyclization. Rat brain membrane binding studies with [^{3}H]naloxone and [^{3}H](D-Ala^2,D-Leu^5)-enkephalin as radiolabels revealed a moderate preference of both analogs for μ-receptors over δ-receptors. Furthermore, the cystine-containing analogs were shown to be highly resistant to enzymatic degradation.

INTRODUCTION

Small linear polypeptides like the enkephalins (1) are flexible molecules and their biologically active conformation may only be realized in the complex with their receptor(s). Manipulation of the peptide-receptor interaction can be achieved by introduction of conformational restrictions into the peptide which could lead to a change in pharmacological properties (e.g. enhanced potency or antagonism). Furthermore, in a situation of receptor heterogeneity the various subclasses of receptors may have different conformational requirements and the incorporation of conformational constraints in their ligands may result in preferential binding to a specific receptor type, thereby producing a more specific pharmacological profile.

Conformational restriction can be achieved either by methylation of α-carbon atoms or amide nitrogens of the peptide backbone (2) or by synthesis of analogs containing cyclic elements. In the case of enkephalin the latter approach recently led to the synthesis of cyclic analogs by substitution of a

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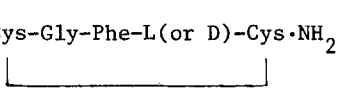

Figure 1. Structure of cystine containing enkephalin analogs.

D-α,ω-diamino acid in position 2 and cyclization of the ω-amino group to the C-terminal carboxyl group of the peptide (3,4). These analogs showed high potency in the bioassay based on inhibition of electrically evoked contractions of the guinea pig ileum (GPI) as well as high resistance to degradation by rat brain membrane peptidases.

In the present paper we describe the synthesis and pharmacological properties of a second class of cyclic enkephalin analogs containing a cystine bridge. Two half cystine residues were substituted in positions 2 and 5 and ring-closure was achieved by oxidative disulfide bond formation (Fig. 1). Whereas D-configuration in position 2 is required (cf. ref. 3) either L- or D-configuration in position 5 can be expected to result in an active compound. Therefore, both [D-Cys^2-L-Cys^5]enkephalinamide (I) and [D-Cys^2-D-Cys^5]-enkephalinamide (II) were synthesized.

MATERIALS AND METHODS

Peptides. Cystine-containing peptides were synthesized by the solid-phase method according to a protocol described elsewhere (5), using a benzhydrylamine resin (Pierce, Rockford, Ill.; 2% cross-linked, 0.21 mM/g titratable amine). α-Amino groups were protected by the tert-butoxycarbonyl group and the benzyl- and p-methylbenzyl (6) group were used for side-chain protection of tyrosine and cysteine, respectively. Peptides were cleaved from the resin and deprotected by treatment with hydrogen fluoride for 90 min at 0°C (20 ml/g resin) in the presence of anisole (1 ml/g resin). Following several washings with ether the crude product was extracted from the resin with 6% acetic acid and the resulting solution was diluted with H_2O to a concentration of approximately 0.3 mM. After adjustment of the pH to 8.0 oxidative disulfide bond formation was achieved by reaction with $K_3Fe(CN)_6$ (2.5-fold excess) for 40 min. Following adjustment of the pH to 5.0 with 50% acetic acid anion exchange resin was added (Amberlite 400, OH^- form) and the suspension was stirred for 20 min. After filtration the absence of free sulfhydryl groups was established with the nitroprusside test. Subsequent volume reduction by evaporation and lyophilization yielded the crude product.

Purification was performed by gel filtration (Sephadex G-15), partition chromatography on Sephadex G-25 with the system butanol-acetic acid-H_2O (4:1:5) and reversed-phase chromatography on a octadecasilyl-silica (ODS) column (7) using a linear gradient of 10-40% methanol in 1% trifluoroacetic acid. Homogeneity of the purified products was demonstrated by high performance liquid chromatography (μ-Bondapak C-18 column (Waters); 5-30% methanol

(linear gradient) in 0.01 M trifluoroacetic acid) and thin layer chromatography (TLC) in the systems: (i) 1-butanol/acetic acid/H_2O (BAW) (4:1:5, organic phase), (ii) 1-butanol/pyridine/acetic acid/H_2O (BPAW) (15:10:3:12), and (iii) sec-butyl alcohol/3% ammonium hydroxide (SH) (100:44). For amino acid analysis peptides were oxidized with H_2O_2/formic acid and hydrolyzed in 6 N HCl for 24 h at 110°C in deaerated tubes and the hydrolysates were analyzed on a Beckman model 121C amino acid analyzer.

[D-Cys^2-L-Cys^5]enkephalinamide (I). TLC Rf: 0.53 (BAW), 0.71 (BPAW), 0.46 (SH). Amino acid analysis: Tyr 1.00, Cys 1.76, Gly 1.01, Phe 1.01.

[D-Cys^2-D-Cys^5]enkephalinamide (II). TLC Rf: 0.53 (BAW), 0.72 (BPAW), 0.53 (SH). Amino acid analysis: Tyr 0.96, Cys 1.98, Gly 1.00, Phe 1.01.

A sample of analog I was oxidized with formic acid/H_2O_2 (9:1) to the cysteic acid derivative, [D-Cys(SO$_3$H)^2,L-Cys(SO$_3$H)^5]enkephalinamide (III) which was purified by ODS chromatography as described above (TLC Rf: 0.24 (BAW), 0.42 (BPAW), 0.23 (SH)). A sample of analog II was reduced with β-mercaptoethanol in 2N acetic acid (compound IV). Due to rapid re-oxidation the latter compound could not be identified by TLC. The Sandoz-compound FK-33'824 was purchased from Peninsula Laboratories, San Carlos, Ca.

In vitro assays. The assay based on inhibition of electrically induced contractions of the GPI (8) was performed as described elsewhere (9). A log-dose/response curve was determined with [Met^5]enkephalin as standard for each ileum preparation and IC50-values of the analogs being tested were normalized as described in the literature (10).

Binding studies with rat brain membrane preparations were carried out as reported elsewhere (9). [^{3}H]naloxone and [^{3}H](D-Ala^2,D-Leu^5)enkephalin at respective concentrations of 0.4 nM and 0.96 nM were used as radiolabels. With both radiolabels incubations were performed for 1 h at 0°C. K_i-values were calculated according to Cheng and Prusoff's equation (11) using values of 0.5 nM and 0.8 nM for the dissociation constants of [^{3}H]naloxone and [^{3}H](D-Ala^2,D-Leu^5)enkephalin, respectively.

Peptide degradation studies were carried out as described previously (3) by incubation of 10 μM solutions of analogs I and II and of [Met^5]enkephalin with rat brain membrane suspensions at 37°C. Aliquots were taken at various times of incubation and following destruction of enzymatic activity (12) and centrifugation the supernatants were assayed on the GPI-preparation.

RESULTS AND DISCUSSION

Both cystine-containing analogs were found to inhibit electrically evoked contractions of the GPI at very low concentrations. The effect on the GPI can be assumed to be mediated via opiate receptors, since it was shown to be completely naloxone-reversible. Log-dose/response curves of analogs I and II were parallel to those obtained with [Met^5]enkephalin and [Leu^5]enkephalin. Thus, the mode of receptor binding appears to be similar for the cyclic and linear peptides. The most active analog, [D-Cys^2-D-Cys^5]enkephalinamide is 73 times as potent as [Met^5]enkephalin and more than twice as potent as the Sandoz-compound FK-33'824 in the GPI-assay (Table 1).

Determination of the narcotic agonist activity of the reduced open-chain analog (III) is complicated by the occurrence of very rapid oxidation.

Table 1. Guinea Pig Ileum Assay of Enkephalin Analogs

No.	Analog	IC50 [nM][a]	Relative potency ([Met^5]enkephalin = 1
I	[D-Cys^2-L-Cys^5]enkephalinamide	1.51 ± 0.03	37.9 ± 0.8
II	[D-Cys^2-D-Cys^5]enkephalinamide	0.78 ± 0.01	73.3 ± 0.9
III	[D-Cys^2-D-Cys^5]enkephalinamide (reduced)	0.99 ± 0.04	57.8 ± 2.3
IV	[D-Cys(SO_3H)^2,L-Cys(SO_3H)^5]enkephalinamide	636 ± 141	0.09 ± 0.02
V	FK-33'824 (Sandoz-compound)	1.72 ± 0.38	33.3 ± 7.3
VI	[Met^5]enkephalin	57.2 ± 6.0	1
VII	[Leu^5]enkephalin	246 ± 39	0.23 ± 0.04

[a] Mean of three determinations ± SEM.

Nevertheless, the reduced sample shows a significantly lower potency ratio than its oxidized parent compound (Table 1); however, this value certainly represents a considerable overestimate. In this context comparison with certain structurally related open-chain analogs is also of interest. Thus, the analog [D-Ala^2,Nva^5]enkephalinamide shows only a potency ratio of 13 relative to [Met^5]enkephalin in the GPI-preparation (13) and the compound [D-Abu^2,Leu^5]enkephalin is only twice as potent (unpublished results). On the basis of these comparisons and the observed lower potency of the reduced analog (III) it can be concluded that the conformational constraints in analogs I and II produced by disulfide bond formation are responsible for their extremely high potencies. This conformational restriction either forces the peptide into a single new conformation with high receptor affinity or enhances the population of a high-affinity conformer in the possible situation of a conformational equilibrium. A parallel observation had been made with the cyclic enkephalin analog Tyr-cyclo[-N$^{\gamma}$-A_2bu-Gly-Phe-Leu-] which was more

Table 2. Comparison of Affinities for μ- and δ-Receptors

o.	Analog	Relative affinity[a,b] [^{3}H]naloxone	Relative affinity[a,b] [^{3}H](D-Ala^2,D-Leu^5)enk.	K_i^δ/K_i^μ
	[D-Cys^2-L-Cys^5]enkephalinamide	1.95 ± 0.46	0.83 ± 0.24	2.55
I	[D-Cys^2-D-Cys^5]enkephalinamide	3.53 ± 0.39	1.63 ± 0.55	2.36
I	[Met^5]enkephalin	1	1	1.09
II	[Leu^5]enkephalin	0.38 ± 0.06	1.42 ± 0.17	0.29

[a] Mean of three determinations ± SEM. [b] IC50-values of [Met^5]enkephalin for displacement of [^{3}H]naloxone and [^{3}H](D-Ala^2,D-Leu^5)enkephalin are 18.3 ± 3.9 nM and 25.1 ± 3.0 nM, respectively.

potent in the GPI-assay than its corresponding open-chain analog (3). The relatively low potency of the oxidized analog IV (Table 1) is likely to be due to the acidic nature of the side-chains in positions 2 and 5.

In order to detect a possible preference of the cystine-containing analogs for either μ- or δ-receptors (14) their ability to displace a μ-receptor specific radiolabel ([^{3}H]naloxone) or a δ-receptor specific radioligand ([^{3}H](D-Ala^2,D-Leu^5]enkephalin) was determined in parallel binding assays. In both assays analog II was found to be about twice as potent as analog I (Table 2) in good agreement with the potency ratio between the two compounds observed in the GPI-assay. Relative to [Met^5]enkephalin both cyclic analogs showed higher potency in displacing [^{3}H]naloxone than in competing with [^{3}H](D-Ala^2,D-Leu^5)enkephalin. The computed ratios of the K_i-values obtained in the two binding assays indicate a moderate preference of both cystine-containing analogs for μ-receptors over δ-receptors.

No enzymolysis was detected with analogs I and II in the degradation experiment at incubation times of up to 1 h. Under the same conditions [Met^5]enkephalin was rapidly degraded as previously reported (12). The high

resistance of the cystine-containing analogs to enzymatic degradation increases their potential value for in vivo studies.

Analogs I and II contain a 14-membered ring structure as does the cyclic analog Tyr-cyclo[-N^{γ}-A_2bu-Gly-Phe-Leu-] (3). However, in the latter compound the ring structure is more rigid than in the cystine-containing analogs due to the presence of an additional planar peptide bond. This difference in the flexibility of the cyclic portions between the two types of analogs is reflected in their different conformational possibilities. Whereas various proposed hydrogen-bonded structures, including the 4 $\longrightarrow$ 1 hydrogen-bonded β_1-bend (15), the 5 $\longrightarrow$ 2 hydrogen-bonded β_1-turn (16,17) and the β-turn with two antiparallel hydrogen bonds between Tyr^1 and Phe^4 (18), are incompatible with the conformational possibilities of Tyr-cyclo[-N^{γ}-A_2bu-Gly-Phe-Leu-], their realization is possible with the cystine-containing analogs.

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REFERENCES

1. Hughes, J., Smith, T.W., Kosterlitz, H.W., Fothergill, L.A., Morgan, B.A., and Morris, R.H. (1975) Nature (London) 258, 577-579.
2. Marshall, G.R., Bosshard, H.E., Vine, W.H., Glickson, J.D., and Needleman, P. (1974) Recent Advances in Renal Physiology and Pharmacology, Wesson, L.G., and Fanelli, Jr., G.M., eds., University Park Press, Baltimore, Md., pp. 215-256.
3. DiMaio, J., and Schiller, P.W. (1980) Proc. Natl. Acad. Sci. USA 77, 7162-7166.
4. Schiller, P.W., DiMaio, J., Lemieux, C., and Nguyen, T.M.-D. (1981) Fed. Proc. 40, 304.
5. Schiller, P.W., Yam, C.F., and Lis, M. (1977) Biochemistry 16, 1831-1838.
6. Erickson, B.W., and Merrifield, R.B. (1973) J. Amer. Chem. Soc. 95, 3750-3756.
7. Bühlen, P., Castillo, F., Ling, N., and Guillemin, R. (1980) Int. J. Peptide Protein Res. 16, 306-310.
8. Paton, W.D.M. (1957) Br. J. Pharmacol. 12, 119-127.
9. Schiller, P.W., Lipton, A., Horrobin, D.F., and Bodanszky, M. (1978) Biochem. Biophys. Res. Commun. 85, 1332-1338.
10. Waterfield, A.A., Leslie, F.M., Lord, J.A.H., Ling, N., and Kosterlitz, H.N. (1979) Europ. J. Pharmacol. 58, 11-18.
11. Cheng, Y.C., and Prusoff, W.H. (1973) Biochem. Pharmacol. 22, 3099-3108.
12. Pert, C.B., Pert, A., Chang, J.K., and Fong, B.T.W. (1976) Nature (London) 194, 330-332.
13. Audigier, Y., Mazarguil, H., and Gros, J. (1980) FEBS Lett. 110, 88-90.

14. Lord, J.A.H., Waterfield, A.A., Hughes, J.S., and Kosterlitz, H.W. (1977) Nature (London) 267, 495-500.
15. Bradbury, A.F., Smyth, D.G., and Snell, C.R. (1976) Nature (London) 260, 165-166.
16. Roques, B.P., Garbay-Jaureguiberry, C., Oberlin, R., Anteunis, M. and Lala, A.K. (1976) Nature (London) 262, 778-779.
17. Jones, C.R., Gibbons, W.A., and Garsky, V. (1976) Nature (London) 262, 779-782.
18. Smith, G.D., and Griffin, J.F. (1978) Science 199, 1214-1216.