A novel side-chain-linked antiparallel cyclic dimer of enkephalin

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The dimeric cyclic enkephalin analog, (H-Tyr-D-Lys-Gly-Phe-Glu-NH_2)$_2$, was isolated as a second major component from the crude product obtained in a solid-phase synthesis of the corresponding cyclic monomer, H-Tyr-D-Lys-Gly-Phe-Glu-NH_2. In comparison with [Leu^5]enkephalin the cyclic dimer is about equipotent in assays representative for μ-opioid receptor interactions and 1/10 as potent at the δ-receptor. The fact that the enkephalin dimer shows a receptor selectivity pattern distinct from that of the cyclic monomer and of the corresponding linear analog suggests that cyclodimerization via side-chain linkages might be generally useful as a means to produce shifts in the activity profiles of peptide hormones and neurotransmitters.

Opioid peptide *Receptor selectivity* *Cyclic enkephalin dimer* *Solid-phase synthesis*

1. INTRODUCTION

Conformational restriction of the opioid peptide enkephalin (H-Tyr-Gly-Gly-Phe-Met(or Leu)-OH) through cyclizations via side-chains resulted not only in highly potent analogs [1,2], but also in compounds showing greatly improved selectivity towards either δ- or μ-opioid receptors [3,4]. In a recently performed synthesis of the cyclic tetrapeptide H-Tyr-D-Orn-Gly-Glu-NH_2 cyclization between the side-chain amino and carboxyl groups of Orn and Glu on the benzhydrylamine resin produced the side-chain-linked antiparallel cyclic dimer (H-Tyr-D-Orn-Gly-Glu-NH_2)$_2$ aside from the cyclic monomer due to intersite reaction [5]. Both the cyclic monomer and the cyclic dimer showed very weak opioid activity in vitro [6]. Here we describe the properties of a side-chain-linked antiparallel cyclic dimer of an enkephalin-related peptide, (H-Tyr-D-Lys-Gly-Phe-Glu-NH_2)$_2$ **(1)** (fig.1), which was identified as a second major component in the crude product resulting from a recently repeated synthesis of the corresponding cyclic monomer, H-Tyr-D-Lys-Gly-Phe-Glu-NH_2 **(2)**. This novel type of peptide dimer contains a 36-membered ring structure and is characterized by a 2-fold symmetry axis. Opioid activities of the cyclic dimer and monomer were compared with those of the corresponding open-chain analog H-

Abbreviations: BAW, *n*-BuOH/AcOH/H_2O (4:1:5, organic phase); Boc, *t*-butoxycarbonyl; BPAW, *n*-BuOH/pyridine/AcOH/H_2O (15:10:3:12); DAGO, H-Tyr-D-Ala-Gly-Phe(NMe)-Gly-ol; DSLET, H-Tyr-D-Ser-Gly-Phe-Leu-Thr-OH; FAB, fast atom bombardment; GPI, guinea pig ileum; MVD, mouse vas deferens

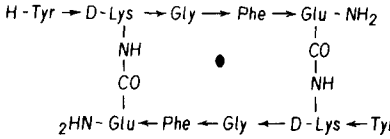

Fig.1. Structural formula of the side-chain-linked antiparallel cyclic dimer of enkephalin, (H-Tyr-D-Lys-Gly-Phe-Glu-NH_2)$_2$ **1**.

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Tyr-D-Nle-Gly-Phe-Gln-NH_2 (**3**) in the GPI assay (μ-receptor representative) and MVD assay (δ-receptor representative) and in binding assays based on displacement of [^{3}H]DAGO (μ-selective) and [^{3}H]DSLET (δ-selective) from rat brain membrane preparations.

2. MATERIALS AND METHODS

The cyclic dimer (**1**) and the cyclic monomer (**2**) were isolated as the 2 major components of a product resulting from a solid-phase synthesis based on a scheme detailed in [5]. HPLC analysis of the crude product indicated that during the cyclization step 60% of the peptide chains had formed the cyclic monomer and 40% had undergone cyclodimerization. The linear analog (**3**) was synthesized by the solid-phase method according to the usual protocol based on the coupling of Boc amino acids (see [2]). The purification and analytical characterization of the cyclic monomer (**2**) have been described [5]. The cyclic dimer (**1**), which had not been isolated before, and the linear analog (**3**) were purified and characterized analogously.

(H-Tyr-D-Lys-Gly-Phe-Glu-NH_2)$_2$ (**1**): TLC R_f; 0.45 (BAW), 0.68 (BPAW). Amino acid analysis; Tyr 1.00, Lys 0.98, Gly 1.01, Phe 1.00, Glu 1.01. FAB mass spectrum; MH^+ (calculated) 1247, (found) 1247.

H-Tyr-D-Nle-Gly-Phe-Gln-NH_2 (**3**): TLC R_f; 0.30 (BAW), 0.67 (BPAW). Amino acid analysis; Tyr 1.00, Nle 1.03, Gly 0.99, Phe 1.00, Glu 1.04.

Binding studies with rat brain membrane preparations were performed as described [2], using [^{3}H]DAGO (Amersham) and [^{3}H]DSLET (New England Nuclear) at respective concentrations of 0.71 and 0.98 nM as radioligands. With both radiolabels, incubations were performed for 2 h at 0°C to prevent peptide degradation. Binding inhibition constants (K_i) were calculated on the basis of Cheng and Prusoff's equation [7], using values of 1.3 and 2.6 nM for the dissociation constants of [^{3}H]DAGO and [^{3}H]DSLET, respectively. The bioassays based on inhibition of electrically evoked contractions of the GPI and MVD were carried out as reported [2]. A log-dose/response curve was determined with [Leu^5]enkephalin as standard for each preparation and IC_{50} values of the analogs being tested were normalized as described in the literature [8]. K_e values for naloxone as antagonist were determined from the ratio of IC_{50} values obtained in the presence and absence of a fixed naloxone concentration (5 nM) [9].

3. RESULTS AND DISCUSSION

In the μ-receptor representative binding assay ([^{3}H]DAGO displacement) the cyclic dimer is about twice as potent as [Leu^5]enkephalin, about 8-times less potent than the structurally related linear analog (**3**) and 4-times less potent than the cyclic monomer (**2**) (table 1). Interestingly, both the cyclic dimer and the linear correlate have about 10-times lower affinity for the δ-receptor than [Leu^5]enkephalin, as indicated by the results obtained with the [^{3}H]DSLET binding assay, whereas the cyclic monomer binds nearly 4-times more tightly to the δ-receptor than does the natural peptide. Calculation of the ratios of the binding inhibition constants (K_i^δ/K_i^μ) from these data permits an assessment of the opioid receptor selectivity of

Table 1

Binding assays of cyclic and linear enkephalin analogs[a]

No.	Compound	[^{3}H]DAGO		[^{3}H]DSLET		K_i^δ/K_i^μ
		K_i^μ (nM)	Potency ratio	K_i^δ (nM)	Potency ratio	
1	(H-Tyr-D-Lys-Gly-Phe-Glu-NH_2)$_2$	5.33 ± 0.48	1.77 ± 0.16	23.2 ± 1.3	0.109 ± 0.006	4.35
2	H-Tyr-D-Lys-Gly-Phe-Glu-NH_2	1.31 ± 0.21	7.20 ± 1.15	0.690 ± 0.025	3.67 ± 0.13	0.527
3	H-Tyr-D-Nle-Gly-Phe-Gln-NH_2	0.628 ± 0.037	15.0 ± 0.9	23.4 ± 2.8	0.108 ± 0.013	37.3
4	[Leu^5]enkephalin	9.43 ± 2.07	1	2.53 ± 0.35	1	0.268

[a] Mean of 3 determinations ± SE

these compounds (table 1). The ratios indicate a slight preference of the cyclic dimer for μ- over δ-receptors and a more pronounced μ-receptor selectivity in the case of the corresponding open-chain analog (**3**). The cyclic monomer is essentially non-selective, as reported in [6].

In the GPI assay (μ-receptor representative) and MVD assay (δ-receptor representative) the potency relationships observed with compounds **1**, **3** and **4** are very similar to those seen in the μ- and δ-receptor-selective binding assays (table 2). The fact that **2** is much more potent in the GPI assay than would be expected on the basis of its affinity for μ-receptors may be explained with an increased 'efficacy' ('intrinsic activity') of this compound [6]. Since the effects of all analogs on the GPI and MVD preparations were readily reversed by naloxone, it is clear that these compounds act via opioid receptors. The K_e values for naloxone as antagonist determined with peptides **1**–**4** in the GPI assay range from 1.25 to 1.59 nM (table 3). These values are typical for μ-receptor interactions [10] and rule out a possible additional involvement of κ-receptors which are also present in this tissue.

It has recently been demonstrated that μ- and δ-opioid receptors differ from one another in their conformational requirements [11]. In view of this observation it seems plausible that the differences in receptor affinity and selectivity observed between analogs **1**, **2** and **3** are due to the various kinds of conformational constraints introduced in the cyclic monomer and dimer through ring formation. Since the cyclic dimer and its linear correlate (**3**) are equipotent in the [^{3}H]DSLET binding assay, it is obvious that the conformational restrictions present in the dimer do not greatly affect the affinity for the δ-receptor. On the other hand, compound **1** is significantly less potent than the open-chain analog in the [^{3}H]DAGO binding assay. This drop in affinity for the μ-receptor may be due to the specific conformational constraints existing in the dimer. Thus, it is conceivable that the conformation(s) of the individual enkephalin chains in the dimer might, to some extent, be induced by hydrogen bonding between 2 moieties on opposite chains. In this context it is of interest to point out that in one of the crystal forms of [Leu^5]enkephalin the peptide chains are aligned in an antiparallel fashion (β-pleated sheet) and linked by inter-chain hydrogen bonds [12]. Alternatively, it can be argued that the optimal interaction of one of the dimer's individual enkephalin chains with the μ-receptor might be impeded by the presence of the second covalently linked chain which could cause steric interference or interact unfavorably with accessory binding sites. The conformational constraints present in the cyclic monomer are clearly different from those in the dimer and can be assumed to be directly responsible for the high affinity of compound **2** for both μ- and δ-receptors.

The properties of several non-cyclic enkephalin dimers have been reported [13–17]. Dimers resulting from linking 2 enkephalin molecules via their C-terminal carboxyl groups to linear spacers of various lengths showed preference for δ- over μ-receptors [16]. It has been suggested that the δ-receptor selectivity of these dimers might be due to interaction with a bivalent δ-binding site [17]. However, in the light of the results of the present study it could also be argued that the 2 enkephalin chains in a folded form of the dimer might interact with each other non-covalently such as to generate a conformation more compatible with the δ-receptor than with the μ-receptor. Thus, the observed δ-receptor selectivity could be due to a

Table 2

GPI and MVD assay of enkephalin analogs[a]

No.	Compound	GPI		MVD		MVD/GPI IC_{50} ratio
		IC_{50} (nM)	Potency ratio	IC_{50} (nM)	Potency ratio	
1	(H-Tyr-D-Lys-Gly-Phe-Glu-NH_2)$_2$	499 ± 94	0.493 ± 0.093	128 ± 22	0.0891 ± 0.0153	0.257
2	H-Tyr-D-Lys-Gly-Phe-Glu-NH_2	1.13 ± 0.14	218 ± 27	0.648 ± 0.132	17.6 ± 3.6	0.573
3	H-Tyr-D-Nle-Gly-Phe-Gln-NH_2	9.08 ± 0.70	27.1 ± 2.1	82.6 ± 16.8	0.138 ± 0.028	9.10
4	[Leu^5]enkephalin	246 ± 39	1	11.4 ± 1.1	1	0.0463

[a] Mean of 3 determinations ± SE

Table 3
Sensitivities to naloxone (K_e) of enkephalin analogs in the GPI assay[a]

No.	Compound	K_e (nM)
1	(H-Tyr-D-Lys-Gly-Phe-Glu-NH_2)$_2$	1.29 ± 0.06
2	H-Tyr-D-Lys-Gly-Phe-Glu-NH_2	1.47 ± 0.20
3	H-Tyr-D-Nle-Gly-Phe-Gln-NH_2	1.59 ± 0.41
4	[Leu^5]enkephalin	1.53 ± 0.43

[a] Mean of 3 determinations ± SE

conformational effect rather than to a cross-linking effect.

Compound **1** represents the first example of a biologically active cyclic peptide dimer of the antiparallel type obtained through amide bond formation between side-chains. Antiparallel cyclic dimers resulting from disulfide bond formation between half-cystine residues have been characterized as side-products in syntheses of lysine-vasopressin [18] and oxytocin [19]. Compared to the cyclic monomers these dimers were found to be significantly less potent. However, in the case of lysine-vasopressin testing in various assay systems revealed interesting differences between the cyclic monomer and dimer in the overall selectivity profile [18]. An antiparallel cyclic dimer of the human atrial natriuretic polypeptide, β-hANP, has recently been isolated from an atrial extract and shown to possess considerable diuretic and natriuretic activity [20]. In all these cases, however, the possibility of disulfide reduction and subsequent re-oxidation resulting in cyclic monomers cannot be entirely excluded. In contrast to the cystine-linked cyclic dimers the antiparallel cyclic enkephalin dimer described in this paper is structurally very stable and its distinct opioid activity pattern indicates that cyclodimerization of this type represents a new approach towards manipulating activity profiles of peptide hormones and neurotransmitters.

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