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Role of the conformational element in peptide-receptor interactions

Studies with cyclic opioid peptide analogs

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Biological activity profiles of three different families of cyclic opioid peptide analogs are presented. It is illustrated that conformational constraints introduced through peptide cyclizations can have drastic effects on receptor affinity, selectivity and 'efficacy' ('intrinsic activity'). Conformational studies of cyclic opioid peptides by various physico-chemical techniques have been initiated and have already produced insight into the conformational requirements of the various opioid receptor types. On the basis of the results obtained, conformational restriction of opioid peptides may represent a first promising step towards the goal of developing peptide mimetics.

The opioid peptides [Met^5]enkephalin (H-Tyr-Gly-Gly-Phe-Met-OH) and [Leu^5]enkephalin (H-Tyr-Gly-Gly-Phe-Leu-OH) as well as many of their linear analogs are flexible molecules capable of existing in a number of different conformations of comparably low energy. This conformational flexibility became apparent from numerous studies involving various theoretical and physico-chemical methods. For example, the Monte-Carlo method was used [1] to generate statistical samples of several biologically active [Trp^4]enkephalin analogs for which efficiencies of singlet-singlet energy transfer between the phenol ring of Tyr^1 (donor) and the indole moiety of Trp^4 (acceptor) and the average intramolecular distances between the two aromatic rings had been determined in dilute aqueous solution by fluorescence measurements [2,3]. The average transfer efficiencies and Try^1–Trp^4 intramolecular distances calculated from the statistical samples of the analogs were found to be in very good agreement with those determined experimentally in H_2O. Similarly, satisfactory agreement between average NMR ^3J(NH-C$^\alpha$H) coupling constants calculated from the Monte-Carlo samples and experimental values was also observed [1]. These results suggest that, in aqueous solution, enkephalin and the analogs investigated in the latter study indeed exist in a conformational equilibrium involving a number of different conformers. The same picture of conformational heterogeneity also emerged from the results of other theoretical energy calculations [4–6].

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Abbreviations: Abu, α-aminobutyric acid; A_2bu, α,γ-diaminobutyric acid; DAGO, H-Tyr-D-Ala-Gly-Phe(NMe)-Gly-ol; DSLET, H-Tyr-D-Ser-Gly-Phe-Leu-Thr-OH; GPI, guinea pig ileum; MVD, mouse vas deferens; Nle, norleucine; NOE, nuclear Overhauser enhancement; Pen, penicillamine; Phe(NMe), N^α-methylphenylalanine; Phg, phenylglycine.

A most convincing experimental demonstration of the conformational flexibility of [Leu^5]enkephalin was possible by X-ray diffraction analysis of two different crystal forms of the peptide. In one crystal form (space group A2) a β-bend structure stabilized by two antiparallel hydrogen bonds between Tyr^1 and Phe^4 was observed [7], whereas in crystals belonging to space group $P2_1$ a totally different, extended conformation was determined [8]. Obviously, both the extended conformation and the β-bend structure are of comparably low energy; however, there is no reason to assume that either of these conformations represents the bioactive one at a particular opioid receptor. The outcome of numerous NMR studies carried out with enkephalins and enkephalin analogs in solution also supports the notion of a conformational equilibrium involving both folded and extended structures (for a review, see ref. 9). Only one of the components existing in the conformational equilibrium may closely resemble the receptor-bound conformation. Alternatively, the bioactive conformation of the peptide may only be adopted upon binding to the receptor in a stepwise manner ('zipper' mechanism) [10].

The pharmacologic characterization of various opiates and opioid peptides carried out over the past decade has revealed opioid receptor heterogeneity. It is currently believed that at least three different opioid receptor classes (μ, δ and κ) exist. Unfortunately, it has not yet become possible to correlate a particular receptor class with one or other of the various opioid activities displayed by both opioid peptides and opiates because of the lack of receptor-specific agonists and antagonists. The endogenous mammalian opioid peptides show quite limited receptor selectivity; for example, the enkephalins display some preference for the δ-receptor but also bind to the μ-receptor with somewhat reduced, but still considerable, affinity.

The low receptor selectivity of the enkephalins, and of many of their linear analogs, is most likely a consequence of their high molecular flexibility which permits conformational adaptation to more than one type of opioid receptor topography. In recent years, receptor selectivity has been improved through incorporation of conformational constraints in opioid peptides. Various types of peptide cyclizations drastically reduce the overall conformational space available to the peptide. One of the first cyclic enkephalin analogs was obtained through substitution of D-α,ω-diaminobutyric acid (A_2bu) in position 2 of the peptide sequence and subsequent ring closure between the γ-amino group of A_2bu and the C-terminal carboxyl function [11,12]. The resulting cyclic analog H-Tyr-cyclo[-D-A_2bu-Gly-Phe-Leu-] was 17-times more potent than [Leu^5]enkephalin in the GPI assay, stable against enzymatic degradation and moderately μ-receptor selective. Parallel pharmacologic characterization of the cyclic peptide and of the corresponding open-chain analog, H-Tyr-D-Abu-Gly-Phe-Leu-NH_2 (Abu = α-aminobutyric acid), revealed that the conformational restriction resulting from ring closure was indeed the cause of the μ-receptor selectivity of the cyclic peptide. Furthermore, these results also led to the conclusion that μ- and δ-opioid receptors have different conformational requirements. Homologs of this cyclic

Table 1

Binding assays of cyclic and linear opioid peptide analogs

No.	Compound	[^{3}H]DAGO	[^{3}H]DSLET	K_i^δ/K_i^μ
		K_i^μ (nM) [a]	K_i^δ (nM) [a]	
1	H-Tyr-cyclo[-D-Lys-Gly-Phe-Leu-]	5.58 ±0.39	76.2 ± 0.7	13.7
2	H-Tyr-D-Orn-Phe-Asp-NH_2	10.4 ±0.7	2220 ±65	213
3	H-Tyr-D-Ala-Gly-Phe(NMe)-Gly-ol	1.22 ±0.12	1280 ±90	1050
4	H-Tyr-D-Lys-Gly-Phe-Glu-NH_2	1.31 ±0.21	0.690± 0.025	0.527
4a	H-Tyr-D-Nle-Gly-Phe-Gln-NH_2	0.628±0.037	23.4 ± 2.8	37.3
5	[Leu^5]enkephalin	9.43 ±2.07	2.53 ± 0.35	0.268

[a] Mean of 3 determinations ± S.E.

Table 2

GPI and MVD assay of cyclic and linear opioid peptide analogs

No.	Compound	GPI	MVD	IC_{50}(MVD)/ IC_{50}(GPI)
		IC_{50} (nM) [a]	IC_{50} (nM) [a]	
1	H-Tyr-cyclo[-D-Lys-Gly-Phe-Leu-]	4.80 ± 1.79	141 ± 28	29.4
2	H-Tyr-D-Orn-Phe-Asp-NH_2	36.2 ± 3.7	3880 ± 840	107
3	H-Tyr-D-Ala-Gly-Phe(NMe)-Gly-ol	28.3 ± 3.7	950 ± 269	33.6
4	H-Tyr-D-Lys-Gly-Phe-Glu-NH_2	1.13 ± 0.14	0.648 ± 0.132	0.573
4a	H-Tyr-D-Nle-Gly-Phe-Gln-NH_2	9.08 ± 0.70	82.6 ± 16.8	9.10
5	[Leu^5]enkephalin	246 ± 39	11.4 ± 1.1	0.0463

[a] Mean of 3 determinations ± S.E.

peptide containing amino acid residues with different side-chain length (D-diaminopropionic acid, D-ornithine or D-lysine) in the 2-position of the peptide sequence, also displayed moderate μ-receptor selectivity. The binding activity profile of H-Tyr-cyclo[-D-Lys-Gly-Phe-Leu-] (**1**), determined in the binding assays based on displacement of [^{3}H]DAGO (μ-selective) and [^{3}H]DSLET (δ-selective) from rat brain membrane preparations, is presented in table 1. The μ-receptor preference of this compound is indicated by its ratio of the binding inhibition constants ($K_i^\delta/K_i^\mu = 13.7$). The μ-receptor selectivity was confirmed by the results obtained in the GPI assay (μ-receptor representative) and in the MVD assay (δ-receptor representative) (table 2).

In an NMR study performed with [D-Ala^2,Met^5]enkephalin in [$^2H_6$]DMSO, a positive NOE between the methyl groups of Ala^2 and Met^5 was detected [13]. This result indicated a close proximity between the termini of the side chains in positions 2 and 5 of the analog and led to the design of the side-chain–to–side-chain cyclized analog H-Tyr-D-Cys-Gly-Phe-D (or L)-Cys-X (X = NH_2 or OH). Cyclic analogs of this type with a C-terminal carboxamide group were found to be highly active in the in vitro bioassays but nonselective [14], whereas the same peptides with a free C-terminal carboxyl group showed moderate δ-selectivity [15]. Substitution of the two half-cystine residues in H-Tyr-D-Cys-Gly-Phe-D (or L)-Cys-OH with penicillamine residues resulted in compounds showing much improved preference for the δ-receptor [16].

Cyclic lactam analogs of peptides can be prepared through ring formation between the amino and carboxyl side-chain groups of appropriately substituted lysine (or ornithine) and aspartic (or glutamic) acid residues. An example of this type of compound is the cyclic analog H-Tyr-D-Orn-Phe-Asp-NH_2 (**2**) [17] which contains a phenylalanine residue in the 3-position of the peptide sequence, as is the case in the linear opioid peptides dermorphin and β-casomorphin. In the binding assays, compound **2** showed about the same affinity for the μ-receptor as [Leu^5]enkephalin, but its affinity for the δ-receptor was 1000-times lower than that of the natural peptide (table 1). Thus, this cyclic analog displays very high preference for μ-receptors over δ-receptors ($K_i^\delta/K_i^\mu = 213$) but is not quite as μ-selective as H-Tyr-D-Ala-Gly-Phe(NMe)-Gly-ol (DAGO, **3**; $K_i^\delta/K_i^\mu = 1050$). However, in the μ-receptor representative GPI bioassay (table 2) the cyclic tetrapeptide analog H-Tyr-D-Orn-Phe-Asp-NH_2 was found to be about as potent as DAGO, whereas in the μ-receptor representative binding assay ([^{3}H]DAGO displacement) it was 10-times less potent (table 1). Due to the fact that in the δ-receptor representative MVD bioassay H-Tyr-D-Orn-Phe-Asp-NH_2 is 4-times less potent than DAGO, this cyclic tetrapeptide displayed an IC_{50}(MVD)/IC_{50}(GPI) ratio 3-times higher than that of DAGO and, therefore, turned out to be a highly selective μ-agonist.

The cyclic pentapeptide analog H-Tyr-D-Lys-Gly-Phe-Glu-NH_2 (**4**) binds with strong affinity to both μ- and δ-receptors (table 1) and, therefore, does not show receptor selectivity. Interestingly, the corresponding open-chain analog H-Tyr-D-Nle-Gly-Phe-Gln-NH_2 (**4a**) is quite μ-receptor

selective [18]. It thus appears that the 18-membered ring structure in cyclic lactam analog **4** has enough flexibility to permit conformational adaptation to both the μ- and δ-receptor topography. In contrast, the predominant conformation of the linear peptide (**4a**) may not be well tolerated at the δ-receptor. Alternatively, its unrestrained side-chains in positions 2 and 5 of the peptide sequence may participate in initial binding interactions which impede efficient binding to the δ-receptor according to a zipper-type mechanism [10].

As had been the case with cyclic peptides **1** and **2**, cyclic analog **4** was also more potent in the μ-receptor representative GPI bioassay than was expected on the basis of its relative μ-receptor affinity. In comparison with its linear correlate (**4a**), the cyclic analog **4** has about half the μ-receptor affinity but is 8-times more potent in the GPI assay. This observation indicates that the cyclic analogs may have an increased efficacy (intrinsic activity) at the μ-receptor compared to their linear correlates. Thermodynamic considerations would suggest that the cyclic analogs should bind more tightly to the receptor than the corresponding open-chain analogs because the loss of internal rotational entropy upon binding is smaller in the case of the cyclic analogs than in the case of the linear ones. The fact that the opposite is observed might suggest that in comparison with the linear analogs a larger part of the receptor-binding energy of the cyclic analogs may be used to lower the energy requirements for the conformational transition of the receptor in the ground state to its excited state. It has been proposed that the efficacy of a compound is related to the rate at which excited receptors are formed as well as to the final equilibrium between excited receptors and receptors in the ground state [19]. In contrast to the behavior of the cyclic analogs, the relative potencies of numerous linear enkephalin analogs in the GPI assay were found to correlate very well with the relative affinities determined in the [^{3}H]DAGO-binding assay. On the other hand, morphine and morphine-related opiates (levorphanol, butorphanol, etc.) displayed lower potency in the GPI assay than was expected on the basis of their μ-receptor affinities. This finding confirms the observation that morphine is in fact a partial agonist [20], whereas many of the cyclic opioid peptide analogs appear to be superagonists.

Conformational studies with cyclic opioid peptide analogs are meaningful because these semi-rigid compounds can no longer undergo major conformational changes upon binding to the receptor and, therefore, the results obtained might provide some insight into the bioactive conformation at the different receptor types. The μ-selective analog H-Tyr-cyclo[-D-A_2bu-Gly-Phe-Leu-] has been the subject of various conformational investigations. The results of an early theoretical study indicated the existence of a Gly^3-Phe^4 type II′ bend [21]. A more recent theoretical investigation led to the proposal of a β-bend stabilized by a hydrogen bond between the D-A_2bu^2-CO and the Leu^5-NH groups [22]. In disagreement with both these studies, the results of NMR experiments in conjunction with molecular dynamics and energy minimization studies suggested the existence of two transannular hydrogen bonds in H-Tyr-cyclo[-D-A_2bu-Gly-Phe-Leu-], one from Leu^5-NH to Gly^3-CO and one from the D-A_2bu side-chain NH to D-A_2bu-CO [23]. In contrast, the same type of study carried out with the corresponding D-Leu^5 analog, H-Tyr-cyclo[-D-A_2bu-Gly-Phe-D-Leu-], did not reveal any stable intramolecular hydrogen bonds [24]. The results of both the molecular dynamics study and of proton relaxation time measurements revealed that the D-Leu^5 cyclic analog has much greater flexibility than the L-Leu^5 analog and, furthermore, indicated that the Tyr^1 and Phe^3 side chains enjoy more orientational freedom in the D-Leu^5 analog than in the L-Leu^5 diastereomer. The higher flexibility of the ring structure and of the Tyr^1 and Phe^3 side chains of H-Tyr-cyclo[-D-A_2bu-Gly-Phe-D-Leu-] may be the reason for the lack of receptor selectivity observed with this compound, whereas the μ-receptor selectivity displayed by the L-Leu^5 analog may be due to its more rigid structure which prevents adequate conformational adaptation to the δ-receptor topography. The conformations of homologs of H-Tyr-cyclo[-D-A_2bu-Gly-Phe-Leu-] were also investigated. Application of the same theoretical approach as in the case of H-Tyr-cyclo[-D-A_2bu-Gly-Phe-Leu-] [21] revealed that a type II′ bend centered on Gly^3-Phe^4 was

also the characteristic feature of the low-energy conformers of H-Tyr-cyclo[-D-A_2pr-Gly-Phe-Leu-] and H-Tyr-cyclo[-D-Orn-Gly-Phe-Leu-] [25]. On the basis of the results of a ^{1}H-NMR study performed in [$^2H_6$]DMSO, two transannular hydrogen bonds (Leu^5-NH → OC-Gly^3 and Orn^2-NH → OC-Orn^2) were proposed for H-Tyr-cyclo[-D-Orn-Gly-Phe-Leu-], whereas H-Tyr-cyclo[-D-Lys-Gly-Phe-Leu-] appeared to exist in a more flexible conformation stabilized by a Lys^2-NH ··· OC-Gly^3 hydrogen bond [26].

A theoretical conformational analysis of the potent, nonselective cyclic analogs H-Tyr-D-Cys-Gly-Phe-L-Cys-NH_2 and H-Tyr-D-Cys-Gly-Phe-D-Cys-NH_2 led again to the proposal of a Gly^3-Phe^4 type II′ bend for both peptides [27]. Furthermore, comparison of the 14-membered ring structures in these two cyclic peptides with the 14-membered ring of H-Tyr-cyclo[-D-A_2bu-Gly-Phe-Leu-] indicated higher flexibility in the case of the cystine-containing analogs, which may explain their lack of receptor preference. Conformational features of H-Tyr-D-Cys-Gly-Phe-D-Cys-NH_2 were investigated experimentally by fluorescence techniques [28]. Tyrosine fluorescence quantum yield measurements performed in H_2O provided evidence for an intramolecular interaction between the Tyr^1 aromatic ring and the disulfide bridge. Fluorescence energy transfer experiments performed with the equally active H-Tyr-D-Cys-Gly-Trp-D-Cys-NH_2 analog in H_2O yielded an average intramolecular distance between the Tyr^1 and Trp^4 aromatic rings of 9.7 ± 0.2 Å which is close to the mean Tyr^1–Trp^4 distance determined with the same technique for the linear analog H-Tyr-D-Ala-Gly-Trp-Met-OH. The conformations of the two nonselective cystine-containing analogs H-Tyr-D-Cys-Gly-Phe-L-Cys-NH_2 and H-Tyr-D-Cys-Gly-Phe-D-Cys-NH_2 were compared with those of the related δ-selective Pen^2-containing analogs H-Tyr-D-Pen-Gly-Phe-L-Cys-NH_2 and H-Tyr-D-Pen-Gly-Phe-D-Cys-NH_2 in a ^{1}H-NMR study carried out in ^2H_2O [29]. Similar chemical shifts, $d\delta/dT$ values and coupling constants were observed for corresponding penicillamine- and cystine-containing analogs, indicating similar overall conformations. However, the NMR data suggested that, in comparison with the corresponding Cys^2 analogs, the Pen^2 analogs show higher rigidity in the C-terminal part of the molecule, which may be related to their more selective activity profile. The temperature dependencies of the amide proton chemical shifts did not indicate the existence of intramolecular hydrogen bonds in any of these four cyclic analogs. The large chemical shift difference observed for the two penicillamine methyl resonances in both Pen^2 analogs is indicative of a ring current effect due to the tyrosyl aromatic moiety and, as in the case of H-Tyr-D-Cys-Gly-Phe-D-Cys-NH_2 (see above), suggests close proximity between the Tyr^1 aromatic ring and the disulfide moiety. A further NMR study dealt with a set of six penicillamine-containing, cyclic analogs all of which display δ-receptor selectivity [30]. Even though differences in various NMR parameters were observed between individual compounds of the series, it was concluded that all these analogs must contain the crucial pharmacophoric elements in a similar spatial disposition.

Recently, we applied a systematic procedure for the determination of the allowed low-energy conformations of the highly μ-receptor selective cyclic analog H-Tyr-D-Orn-Phe-Asp-NH_2 (**2**), using the software package SYBYL (Tripos Associates, St. Louis, MO) [31]. A comprehensive grid search of the 13-membered ring structure of **2** devoid of the exocyclic Tyr^1 residue and the Phe^3 side chain resulted in only four low-energy conformations. To these four structures, the exocyclic Tyr^1 residue and the Phe^3 side chain were added and an extensive energy minimization performed with each. The results obtained indicated that the Tyr^1 and Phe^3 side chains enjoy considerable orientational freedom, but nevertheless, only a limited number of low-energy side-chain conformations were found. The lowest energy conformation obtained did not contain any intramolecular hydrogen bonds and was characterized by a tilted, stacking arrangement of the two aromatic rings (fig. 1). This conformational analysis has recently been extended to several 13-membered ring cyclic analogs related to **2**, which show considerable diversity in their μ-receptor affinity and selectivity [32]. The two potent and μ-selective analogs H-Tyr-D-Orn-Phe-D-Asp-NH_2 and H-Tyr-D-Asp-Phe-Orn-NH_2 showed a tilted, stacking arrangement of the

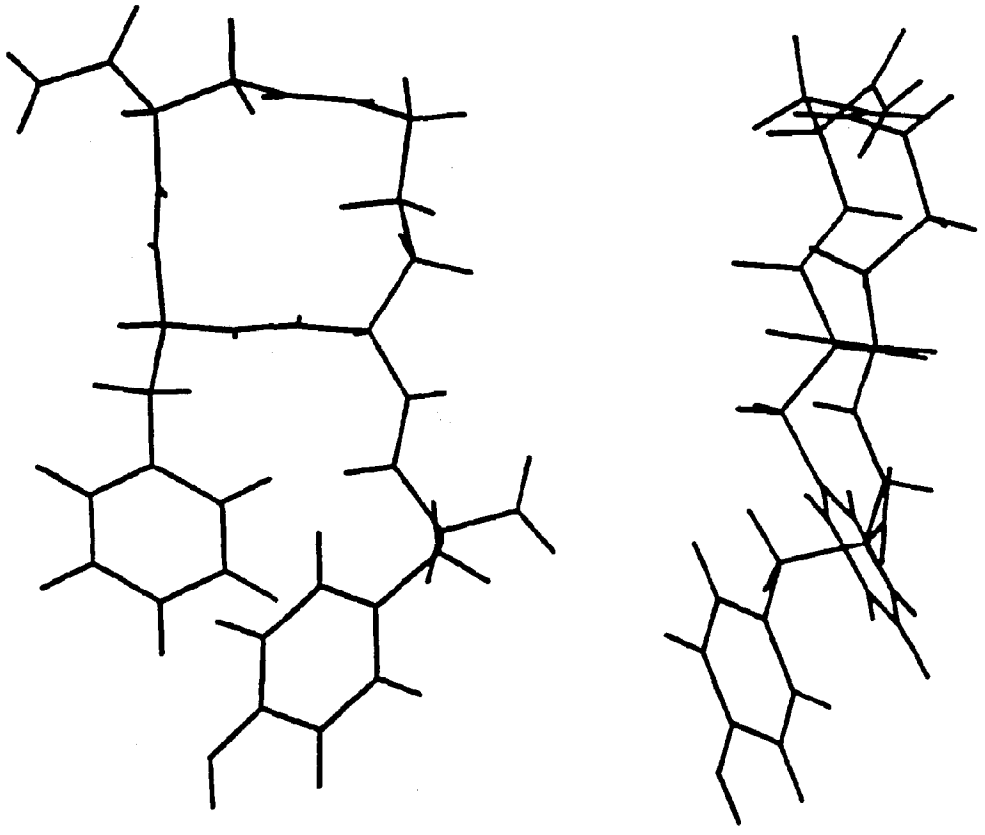

Fig. 1. Molecular graphics display of lowest energy conformer of H-Tyr-D-Orn-Phe-Asp-NH_2 (2) (two different views).

two aromatic rings in their lowest energy conformations, similar to that observed in the lowest energy conformer of **2**. Among the analogs with reduced μ-receptor affinity, H-Tyr-D-Orn-Phg-Asp-NH_2 showed a lowest energy conformation characterized by a fully stacked, parallel arrangement of the two aromatic rings rather than a tilted, stacking interaction. No stacking interaction was observed in low-energy conformers of the weak μ-agonists H-Tyr-D-Orn-Phe(NMe)-Asp-NH_2 and H-Tyr-L-Orn-Phe-Asp-NH_2. Taken together, these results suggest that a specific, tilted, stacking interaction of the aromatic rings in the 1- and 3-positions of cyclic analog **2** may represent an important structural requirement for binding at the μ-receptor.

In conclusion, these studies have shown that conformational restriction through various side-chain–to–side-chain or side-chain–to–end-group cyclizations permits the manipulation of receptor affinity, receptor selectivity and the efficacy of opioid peptides in a more effective manner than can be achieved through more conventional analog design based on simple amino acid substitutions. The further conformational analysis of such cyclic analogs can be expected to provide a rational basis for the design of even more selective opioid receptor ligands and, ultimately, may lead to the development of peptide mimetics.

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