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A cyclic enkephalin analog with high *in vitro* opiate activity

(opioid peptides/peptide synthesis/enzymatic degradation/conformationally restricted enkephalin analog/ receptor-bound conformation)

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ABSTRACT **A conformationally restricted analog of [Leu^5]enkephalin was synthesized by cyclization of the COOH-terminal carboxyl group of leucine to the γ-amino moiety of α,γ-diaminobutyric acid (A_2bu) substituted in position 2 of the peptide. Relative to [Leu^5]enkephalin, the cyclic analog with D configuration in position 2, H-Tyr-*cyclo*(-N^γ-D-A_2bu-Gly-Phe-Leu-), was 17.5 times more potent in the guinea pig ileum assay and twice as potent in the rat brain receptor binding assay, whereas its diastereomer H-Tyr-*cyclo*(-N^γ-L-A_2bu-Gly-Phe-Leu-) showed low activity. The cyclic D isomer was also slightly more active than the open-chain reference compound [D-Nva^2, Leu^5]enkephalinamide in both assays, and it proved to be highly resistant to degradation by brain "enkephalinases." The steric constraints introduced in H-Tyr-*cyclo*(-N^γ-D-A_2bu-Gly-Phe-Leu-) were shown to prevent the realization of most of the conformational features ascribed to linear enkephalin in solution or in the crystalline state and permitted an assessment of proposed models of the conformation of enkephalin when it is bound to the receptor.**

The ability of the endogenous opioid peptide enkephalin [Tyr-Gly-Gly-Phe-Met(or Leu)] and of morphine and its derivatives to bind to the same receptors (1) is likely to be based on structural similarities between the two classes of compounds. Correspondence between critical chemical functions in the peptide and in opiate alkaloids has been established on the basis of structure–activity data obtained with enkephalin analogs. In particular, the tyramine moieties in enkephalin and in morphine (see Fig. 1) have been shown to play analogous roles in the interaction with opiate receptors (cf. ref. 2). On the other hand, a possible correspondence of the amino acid residues in positions 4 and 5 of the pentapeptide to structural elements of morphine and its surrogates is still a matter of debate.

Because the spatial disposition of the important chemical groups is defined in the semirigid opiate alkaloids, conformational studies of enkephalin are of particular interest and relevance. Both theoretical and experimental approaches have led to the proposition of various conformational models for enkephalin in unsolvated form, in the crystalline state, in solution, and when bound to its receptor (cf. ref. 3). Little consensus about the backbone conformation and the degree of conformational heterogeneity of enkephalin in aqueous solution has been reached to date. Furthermore, the question whether enkephalin binds to receptors by virtue of a molecular shape predetermined in solution or whether the receptor-bound conformation is realized only after adaptation to the geometrical requirements of the receptor binding site cannot be easily answered owing to the inherent flexibility of the peptide molecule. However, it is possible to approximate the receptor-bound conformation by synthesis and pharmacological evaluation of conformationally restricted enkephalin analogs.

Because introduction of a side chain with D configuration in position 2 is well tolerated (4, 5), an analog with D-α,γ-diaminobutyric acid (D-A_2bu) in position 2 was synthesized and conformational restriction was achieved by cyclization of the γ-amino group of D-A_2bu to the COOH-terminal carboxyl group (Fig. 2). The resulting compound, H-Tyr-*cyclo*(-N^γ-D-A_2bu-Gly-Phe-Leu-) (**Ia**), and its diastereomer with L configuration in position 2 (**Ib**) were tested in the rat brain opiate receptor binding assay and in the guinea pig ileum (GPI) bioassay. For comparative purposes the corresponding open-chain analogs of [Leu^5]enkephalinamide with D- and L-norvaline (Nva) in position 2 (**IIa** and **IIb**) were also synthesized and tested *in vitro*. In the design of the latter analogs Nva in position 2 was chosen over A_2bu because substitution of the latter residue would introduce an additional positive charge, which is likely to interfere with the receptor interaction.

Natural enkephalins are rapidly degraded by various aminopeptidases, carboxypeptidases, and endopeptidases present in brain. Substitution of D-alanine in position 2 of the enkephalin sequence and amidation of the COOH-terminal carboxyl group drastically reduced enzymatic hydrolysis (4) but did not completely eliminate it, as shown in a more recent study (6). Furthermore, the results of the latter investigation suggested that conformational parameters might influence the specificity of peptidases involved in the metabolism of enkephalin and its analogs. Because in analog **Ia** the residue in position 2 is in the D configuration and the COOH-terminal carboxyl group is engaged in an amide bond, it can be expected to be highly resistant to enzymolysis. Furthermore, the conformational constraint introduced in the cyclic analog may further favorably alter its degradative profile as compared to the corresponding open-chain analog **IIa**. Therefore, a comparative study on the degradation of compounds **Ia** and **IIa** by "enkephalinases" located in rat brain membranes will also be presented in this paper.

MATERIALS AND METHODS

Peptide Synthesis. The cyclic analogs were synthesized by a combination of solid-phase and solution methods. N^α-Tos-N^γ-Boc-diaminobutyric acids with L and D configuration were prepared by tosylation of the corresponding glutamine isomer, subsequent Hofmann degradation (7), and reaction with Boc-azide. N^α-Tos-D-A_2bu-Gly-Phe-Leu-OH was prepared by the solid-phase method, using a 1% cross-linked polystyrene resin with a leucine substitution of 0.50 mmol/g of resin. A 2-mol excess of Boc-amino acid and 1-ethoxycarbonyl-2-ethoxy-

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Abbreviations: A_2bu, α,γ-diaminobutyric acid; Nva, norvaline; Tos (tosyl), 4-toluenesulfonyl; Boc, *tert*-butoxycarbonyl; Z, benzyloxycarbonyl; EEDQ, 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline; TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography; PEO, 7α-[(*R*)-1-hydroxy-1-methyl-3-phenylpropyl]-6,14-*endo*-ethenotetrahydrooripavine; GPI, guinea pig ileum; CPK, Corey–Pauling–Koltung.

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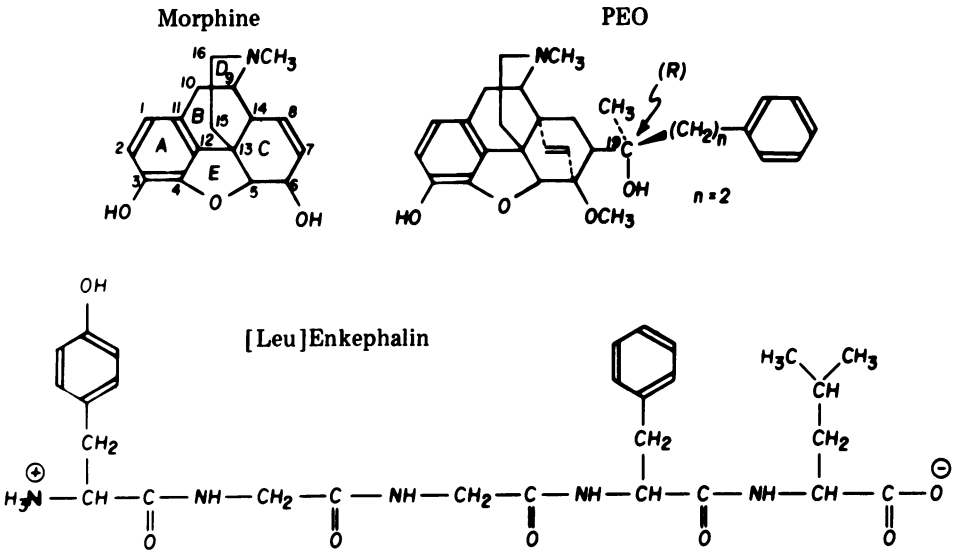

FIG. 1. Structural formulas of morphine, 7α-[(*R*)-1-hydroxy-1-methyl-3-phenylpropyl]-6,14-*endo*-ethenotetrahydrooripavine (PEO), and [Leu^5]enkephalin.

1,2-dihydroquinoline (EEDQ) (8) were used in the coupling steps. The protocol of synthesis was the following: (*1*) CH_2Cl_2, 1 min three times, EtOH, 1 min; (*2*) trifluoroacetic acid [50% (vol/vol) in CH_2Cl_2], 1 hr; (*3*) CH_2Cl_2, 1 min three times, EtOH, 1 min; (*4*) diisopropylethylamine [10% (vol/vol) in CH_2Cl_2], 1 hr, EtOH, 1 min; (*5*) CH_2Cl_2, 1 min four times, EtOH, 1 min; (*6*) coupling step of amino acid derivative, 15 hr; (*7*) CH_2Cl_2, 1 min three times, EtOH, 1 min. The tosylated tetrapeptide was cleaved from the resin by reaction (1 hr) with HF at 0°C in the presence of anisole. Cyclization attempts with dicyclohexylcarbodiimide, EEDQ, or the classical azide coupling method were unsuccessful. Cyclization was finally performed in 50% yield with diphenylphosphorazidate (9) in dimethylformamide at high dilution (0.1 mM). Lack of free amine in the resulting major product was demonstrated by failure to react with ninhydrin on a thin-layer chromatography (TLC) plate. After removal of the tosyl group by treatment with sodium in liquid ammonia, the cyclic tetrapeptide was purified by partition chromatography on Sephadex G-25 with the system 1-butanol/acetic acid/H_2O (4:1:5, vol/vol) and high-performance liquid chromatography (HPLC) [μ-Bondapak C_{18} column (Waters); 0.05 M ammonium acetate, pH 6/50% (vol/vol) methanol]. The possibility of cyclodimerization could be ruled out on the basis of a mass spectrum which revealed a molecular ion peak at a mass-to-charge ratio of 417, corresponding to the cyclic monomer. Coupling of Z-L-tyrosine with EEDQ in dimethylformamide, subsequent deprotection by catalytic hydrogenation, and purification by HPLC (conditions as described above) yielded the final product (**Ia**) (overall yield 20%). Analog **Ib** was synthesized by an analogous route in 32% overall yield.

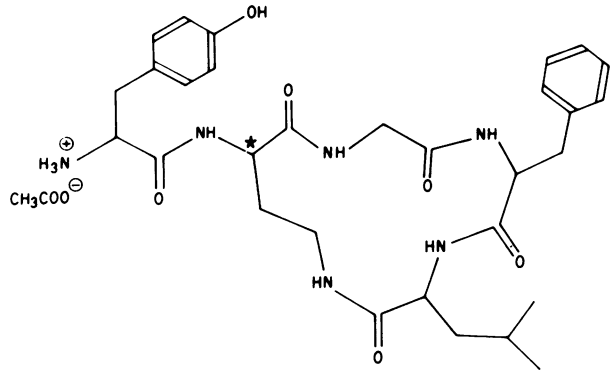

FIG. 2. Structural formula of H-Tyr-*cyclo*(-*N*$^\gamma$-A$_2$bu-Gly-Phe-Leu-). Configuration at the position indicated by the star is D in **Ia** and L in **Ib**.

Analogs **IIa** and **IIb** were prepared by the solid-phase method according to a synthesis program described elsewhere (10) and purified to homogeneity by HPLC under the conditions indicated above. The respective overall yields were 30% and 34%.

Homogeneity of the peptides was verified by HPLC and by ascending TLC on precoated plates (silica gel G, 250 μm, Analtech, Newark, DE) in the following systems (all vol/vol): (*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 hydrolyzed in 6 M HCl for 24 hr at 110°C in deaerated tubes and the hydrolysates were analyzed on a Beckman model 121C amino acid analyzer.

H-Tyr-cyclo(-N$^\gamma$-D-*A$_2$bu-Gly-Phe-Leu*-). TLC R_F: 0.47 (BAW), 0.70 (BPAW), 0.50 (SH). Amino acid analysis: Tyr 0.93, A$_2$bu 1.03, Gly 1.00, Phe 1.00, Leu 1.03.

H-Tyr-cyclo(-N$^\gamma$-L-*A$_2$bu-Gly-Phe-Leu*-). TLC R_F: 0.46 (BAW), 0.69 (BPAW), 0.43 (SH). Amino acid analysis: Tyr 0.93, A$_2$bu 0.91, Gly 1.01, Phe 1.00, Leu 1.04.

H-Tyr-D-Nva-Gly-Phe-Leu-NH$_2$. TLC R_F: 0.63 (BAW), 0.76 (BPAW), 0.44 (SH). Amino acid analysis: Tyr 0.97, Nva 1.09, Gly 1.00, Phe 1.01, Leu 1.04.

H-Tyr-L-Nva-Gly-Phe-Leu-NH$_2$. TLC R_F: 0.61 (BAW), 0.75 (BPAW), 0.51 (SH). Amino acid analysis: Tyr 0.96, Nva 1.01, Gly 1.01, Phe 1.00, Leu 1.00.

***In Vitro* Opiate Activities and Enzymatic Degradation Studies.** Relative opiate receptor affinities were obtained by displacement of [^{3}H]naloxone from rat brain membrane preparations essentially as described in the literature (11). The final concentration of [^{3}H]naloxone was 0.5 nM and incubations were performed for 1 hr at 0°C in order to minimize enzymatic peptide hydrolysis. Stereospecific binding as determined by displacement of [^{3}H]naloxone with excess (10 μM) [Met^5]enkephalin accounted for 70–80% of total binding. Further details of the binding assay have been reported elsewhere (2).

Narcotic agonist activities were determined with an assay

based on inhibition of electrically induced contractions of the GPI (12). Single pulses of 4-ms duration were delivered with voltages ranging from 3 to 6 V and isometric contractions were recorded. Other details of the GPI assay have been reported (13).

For degradation studies, rat brain membrane suspensions were prepared by a procedure analogous to that used in the binding assay except for an additional washing with standard buffer (50 mM Tris·HCl, pH 7.7) at 4°C after incubation at 37°C. The final suspension contained 30 mg of membranes (wet weight) per ml of buffer. In a control experiment the supernatant obtained by centrifugation of this suspension at 2900 × *g* was found to be devoid of opiate activity both in the GPI assay and in the binding assay. One hundred microliters of peptide solution was combined with 900 μl of membrane suspension. Final concentrations of [Leu^5]enkephalin and analogs **Ia** and **IIa** were 100 μM, 10 μM, and 10 μM, respectively. Incubations were performed for 10, 20, 40, and 60 min at 37°C and for 60 min at 0°C. After incubation enzymatic activity was destroyed by dipping the sample tubes for 2 min into boiling water (4). After centrifugation at 2900 × *g*, aliquots of the supernatant were tested for opiate activity in the GPI assay and in the binding assay.

RESULTS AND DISCUSSION

Compared to [Leu^5]enkephalin, the cyclic analog **Ia** is 17.5 times more potent in the GPI assay and nearly twice as active as the corresponding open-chain analog with D-norvaline in position 2 (**IIa**) (Table 1). The high potencies of compounds **Ia** and **IIa** in comparison to [Leu^5]enkephalin are mainly due to their resistance to enzymatic degradation taking place in the ileum preparation. No reduction in the inhibition of the electrically evoked contractions was detected with either analog for up to 4 hr after administration to the organ bath, in contrast to the quick reversal observed with [Leu^5]enkephalin. The cyclic analog **Ia** is a full agonist, because 100% inhibition of the electrically stimulated ileum contractions could be achieved at a concentration of 80 nM. The 80% inhibition of contractions produced by compound **Ia** at a concentration of 40 nM could be completely reversed by addition of the antagonist naloxone (60 nM). This result indicates that the morphinomimetic effect of the cyclic analog is mediated via opiate receptors.

In the rat brain opiate receptor binding assay (Table 1) analog **Ia** was shown to be twice as active as [Leu^5]enkephalin and 20% more potent than the corresponding linear analog (**IIa**). These results reflect true relative affinities, because peptide degradation under the conditions of the binding assay is minimal (see Fig. 3). The potency relationships between analogs **Ia** and **IIa** in the binding assay and in the GPI assay are quite similar, which is compatible with the fact that both assays select for the μ receptor (14).

Parallel log-dose–response curves were observed for [Leu^5]-enkephalin and all analogs investigated both in the binding assay and in the GPI assay. The low potencies obtained with compounds **Ib** and **IIb** are in agreement with the observation that substitution of L amino acid residues in position 2 of enkephalin results in analogs with low opiate activity (15). The fact that inversion of the configuration in position 2 produces the same effect in both analogs, together with the observed parallelism of the log-dose–response curves, strongly suggests that the modes of binding of the cyclic analog and of the corresponding open-chain analog are identical.

Incubation of a [Leu^5]enkephalin solution with extensively washed rat brain membranes at 37°C produced rapid degradation, and opiate activity was almost completely lost after 40 min of exposure to rat brain enkephalinases as monitored in the GPI assay and in the opiate receptor binding assay (Fig. 3). This result is in agreement with the fast metabolism of [Met^5]enkephalin observed in a similar experiment (4). In contrast to the rapid enzymolysis occurring at 37°C, little degradation of [Leu^5]enkephalin is observed after incubation with rat brain membranes for 60 min at 0°C. No metabolism of analogs **Ia** and **IIa** was detected after incubation at 37°C for 60 min. Thus, both analogs are highly resistant to degradation by aminopeptidases, carboxypeptidases, and the dipeptidyl carboxypeptidase enkephalinase (16) under these assay conditions. This result confirms the importance of a free carboxyl group at the COOH terminal for the interaction with the active site of enkephalinase (17). Obviously, the high stability of the cyclic analog is of interest in relation to *in vivo* analgesic tests, which will be the subject of a future publication. Because analogs **Ia** and **IIa** are equally stable against enzymatic degradation, the increased potency of the cyclic analog as compared to the open-chain analog has to be accounted for by conformational factors. Cyclization might produce a single new conformation with enhanced affinity for opiate receptors. Alternatively, several different conformers might coexist in solution and the conformational restrictions introduced in analog **Ia** could produce a shift in the conformational equilibrium resulting in an increased population of a particular high-affinity conformation.

Even though cyclization does not produce a single unique conformation, the number of possible backbone conformations in compound **Ia** is drastically reduced. In particular, *all* possible conformations are characterized by an extremely tight ring structure. This is illustrated in Fig. 4 which shows a Corey–Pauling–Koltung (CPK) model of compound **Ia** in a plausible conformation with all *trans* amide bonds in the cyclic tetrapeptide segment. It is of interest to analyze the conformational possibilities of analog **Ia** in relationship to conformational features that have been proposed for native enkephalin in various states. A β_I bend stabilized by a hydrogen bond between the amino group of Phe^4 and the carbonyl group of Tyr^1 had been proposed for [Met^5]enkephalin on the basis of theoretical

Table 1. *In vitro* opiate activities of enkephalin analogs

No.	Analog	Binding assay*† Relative potency‡	GPI assay* IC_{50}, nM	GPI assay* Relative potency‡
Ia	H-Tyr-*cyclo*(-N^γ-D-A_2bu-Gly-Phe-Leu-)	2.0 ± 0.5	9.02 ± 1.83	17.5 ± 3.6
Ib	H-Tyr-*cyclo*(-N^γ-L-A_2bu-Gly-Phe-Leu-)	0.010 ± 0.003	903 ± 204	0.17 ± 0.04
IIa	H-Tyr-D-Nva-Gly-Phe-Leu-NH_2	1.67 ± 0.24	14.9 ± 1.0	10.6 ± 0.7
IIb	H-Tyr-L-Nva-Gly-Phe-Leu-NH_2	<0.01	>40,000	<0.004
III	H-Tyr-Gly-Gly-Phe-Leu-OH	1	158 ± 56	1

* Mean of three experiments ± SEM.

† The concentration at which stereospecific [^{3}H]naloxone binding was 50% inhibited, IC_{50}, was 40–70 nM for [Leu^5]enkephalin in this assay system.

‡ Potency relative to [Leu^5]enkephalin (= 1).

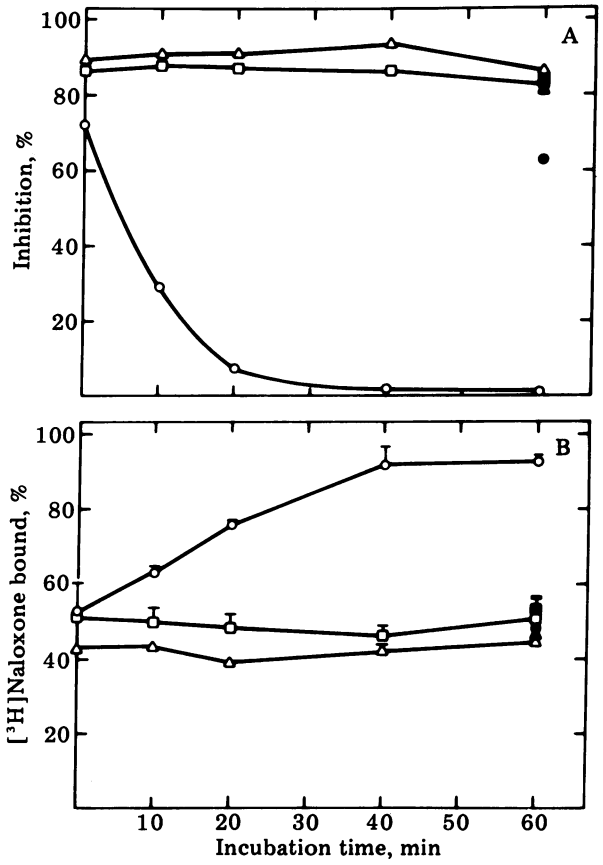

FIG. 3. Enzymatic degradation of [Leu⁵]enkephalin (○), analog **Ia** (△), and analog **IIa** (□) by incubation with rat brain membranes at 37°C (open symbols) and at 0°C (closed symbols). Remaining opiate activity after various times of incubation was determined with the GPI assay (*A*) and with the opiate receptor binding assay (*B*).

considerations (18). Inspection of a CPK model shows that formation of this hydrogen bond is still possible in analog **Ia**. However, due to steric constraints, the resulting β_I bend is significantly distorted in comparison to the β_I-turn structure described by Venkatachalam (19). A β bend stabilized by two hydrogen bonds between the amino nitrogen of Tyr¹ and the carbonyl oxygen of Phe⁴ and between the amino nitrogen of Phe⁴ and the carbonyl oxygen of Tyr¹ is observed in the crystal structure of [Leu⁵]enkephalin (20). Formation of these two antiparallel hydrogen bonds is not possible in *any* conformation of the cyclic analog. On the basis of nuclear magnetic resonance studies performed in dimethyl sulfoxide a 5 → 2 hydrogen-bonded β_I bend stabilized by a salt bridge between the terminal amino and carboxy groups had been proposed for [Met⁵]enkephalin (21, 22). In analog **Ia** the residues in positions 2 and 5 are contained in the cyclic portion of the peptide and formation of a linear transannular 5→2 hydrogen bond is impossible due to the tightness of the ring. Furthermore, the terminal amino and carbonyl groups are separated by at least 8 Å in any possible conformation of the cyclic analog.

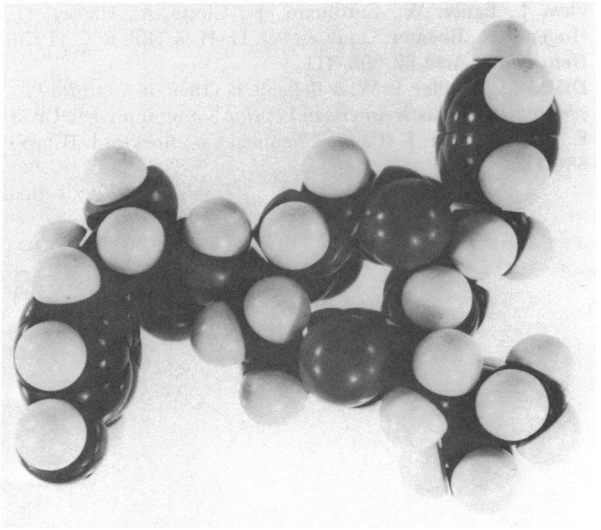

FIG. 4. CPK model of H-Tyr-*cyclo*(-N^{γ}-D-A_2bu-Gly-Phe-Leu-). The tyrosine side chain is oriented in analogy to the steric situation in PEO.

It thus appears that most of the conformational features proposed for enkephalin in the crystalline state or in solution cannot be realized in the conformationally restricted analog **Ia**. This finding does not necessarily imply that the proposed crystal structure or solution conformations are wrong, because the conformation of enkephalin when bound to the receptor need not be identical with its conformation in the crystal or in solution. In fact, the slow association and dissociation observed with enkephalin in receptor binding studies as compared to rigid opiate alkaloids has been interpreted in favor of a conformational change occurring during the binding process (23).

On the other hand, the high opiate activity observed with analog **Ia** permits some unequivocal statements with regard to proposed models of the receptor-bound conformation of native enkephalin under the reasonable assumption that the linear and the cyclic peptide have identical modes of binding. According to one proposition (18) the aromatic ring of Phe⁴ corresponds to the phenylethyl substituent on carbon 19 of the potent morphine derivative PEO (see Fig. 1). In the β_I-bend models of enkephalin the spatial relationship between Tyr¹ and the aromatic ring of Phe⁴ is similar to that between the tyramine moiety and the phenylethyl substituent on carbon 19 of the semirigid PEO (phenol–phenyl distance ≈ 10 Å). Therefore, it was suggested that the 4→1 and 5→2 hydrogen-bonded β_I-bend models might represent the receptor-bound conformation of enkephalin (18, 22). The results of fluorescence studies with the biologically active 4-tryptophan analog of [Met⁵]enkephalin in dilute aqueous solution had indicated the existence of folded conformations with an average intramolecular distance of approximately 10 Å between the aromatic rings in positions 1 and 4 (10). However, it was also demonstrated that the observed folded conformation(s) need not be stabilized by 4→1 or 5→2 hydrogen bonds between amide nitrogen and carbonyl groups of the peptide backbone (24). In agreement with the latter study, inspection of the CPK model of compound **Ia** shown in Fig. 4 reveals that superposition of the tyramine group and the phenyl ring upon the corresponding moieties in PEO is possible, even though this conformation is devoid of the proposed hydrogen bonds. Therefore, the correspondence between the side chain of phenylalanine in enkephalin and the phenylethyl substituent in PEO still holds in the light of the present study, whereas hydrogen-bonded β bends are unlikely structural elements of the receptor-bound conformation. The latter conclusion is in agreement with the high activity observed with analogs carrying a methyl group on the amide nitrogen of residue 4 or 5 (cf. ref. 24).

In an alternative proposal (25) it has been suggested that in addition to the tyramine moiety, carbons 5 and 6 of the C ring of morphine constitute an important structural element for the interaction with opiate receptors and that the carbons in *para* and *meta* positions of the aromatic ring of Phe⁴ might play a corresponding role in enkephalin. Based on superposition of the

tyramine segments in morphine and in enkephalin, a computer search produced an enkephalin conformation accommodating the *meta* carbon of Phe[4] at a position in space that nearly coincides with that of carbon 6 in the C ring of morphine (25). This receptor-bound conformation is characterized by a close proximity (≈5 Å) between the two aromatic rings in positions 1 and 4 of the peptide. However, a study with a CPK model indicates that the backbone structure defined in this conformation does not permit ring closure, because the γ-amino group of D-A_2bu[2] and the COOH-terminal carboxyl group are separated by a gap of at least 5 Å. Thus, our findings are not compatible with this model of the receptor-bound conformation of enkephalin.

Recently, an attempt has been made to freeze the conformation of [Met[5]]enkephalin by head-to-tail cyclization via a 2-aminoethyl bridge (26). The total loss of activity observed with the resulting analog is in agreement with reports indicating that substitution of bulky alkyl groups on the terminal amino group leads to a drastic reduction in activity (27, 28). Furthermore, head-to-tail cyclization also entails enhanced structural rigidity in the tyramine part of the molecule. In the cyclic analog **Ia** the tyrosine residue is retained outside the ring structure and assumes equal flexibility as in the native enkephalins. The importance of structural flexibility in the tyramine region for opiate receptor interaction has recently been demonstrated with piperazinone analogs of enkephalin (29). The latter analogs were found to be completely inactive in the *in vitro* assays due to conformational restrictions in the NH_2-terminal dipeptide segment.

Compound **Ia** has a 14-membered ring structure. Reduction or expansion of the ring size should be feasible by synthesis of cyclic analogs with a shortened (D-α,β-diaminopropionic acid) or lengthened (D-ornithine, D-lysine) side chain in position 2. It will be of considerable interest to assess the effect of these subtle changes in conformational restriction on opiate activity.

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