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Structural Requirements for Specific Recognition of μ or δ Opiate Receptors

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SUMMARY

A comparison of the inhibitory potency of rationally selected new analogues of enkephalins on the evoked contractions of guinea pig ileum (μ receptors) and mouse vas deferens (δ receptors) allowed the definition of the structural components required for specific recognition of μ or δ receptors. A high specificity for μ binding sites is obtained (a) by shortening the enkephalin sequence and removing the terminal carboxyl group; (b) by replacing the aromatic Phe^4 residue with a lipophilic alkyl chain; and (c) by introducing as a second residue a hydrophobic amino acid strictly of the D-configuration whose side-chain probably interacts with a specific μ-receptor subsite. Compared with methionine-enkephalin, such modifications lead to a 100-times loss of potency on mouse vas deferens but a 2-fold enhanced activity on guinea pig ileum. These short peptides display a high folding tendency as shown by ^{1}H-NMR experiments and conformational calculations. The low-energy conformer of the highest μ-specific peptide exhibits a T-shaped structure similar to that of morphine, evidencing the close correspondence of several biologically critical components in both compounds. All of the proposed μ-specific requirements account for the reported variations in the biological activity of various modified enkephalins. The μ-agonist potency of endogenous enkephalins could be related to a fitting of the side-chain of their fifth amino acid in the μ hydrophobic subsite. This feature is precluded in Tyr-Gly-Gly-Phe and could explain its very low potency. The requirements for δ receptor specificity are even more strict and involve (a) an aromatic moiety in the fourth position; (b) a conformational key role of the amino acid(s) following the Phe^4, improving the fitting of the Phe^4 side-chain in a specific δ-receptor subsite; and (c) a lengthening of the enkephalin sequence and the introduction of a hydrophilic side-chain in position 2 which decrease the μ specificity. These modifications lead to peptides almost three orders of magnitude more active on mouse vas deferens than on guinea pig ileum. On the whole, μ receptors bind preferentially highly hydrophobic compounds with compact structures, whereas δ receptors exhibit a stronger affinity for larger peptides with hydrophilic components. These findings obtained at the periphery are corroborated at the level of rat brain receptors by preliminary results from binding experiments. Thus ^{3}H-labeled Tyr-D-Ser-Gly-Phe-Leu-Thr, the most selective δ agonist, interacts exclusively with δ-binding sites at concentrations up to 20 nM. In contrast, Tyr-D-Ala-Gly-NH-CH(CH_3)-CH_2-CH(CH_3)$_2$ exhibits a specificity almost 50 times greater for μ receptors than for δ receptors as shown by competition experiments. This extensive structure-activity study could permit an exploration of the pharmacological significance of μ and δ receptors through a rational design of highly specific ligands.

INTRODUCTION

The presence in brain as well as in peripheral organs of at least two binding sites for enkephalins is now well established (1–3). The high-affinity site called δ, or enkephalin, receptor, exhibits a high preference for peptide structures. The low-affinity site designated μ, or morphine, receptor interacts preferentially with natural and

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synthetic opiates. The μ and δ receptor subtypes are independent as shown by the binding kinetics of enkephalins or opiates which are not significantly modified by the simultaneous presence of the two kinds of substances (2). These two binding sites are not regularly distributed both in brain and at the periphery (2, 4). Thus, the MVD[3] contains a larger population of δ receptors, whereas the GPI is enriched with μ receptors. Moreover, the presence in both organs of a third binding site (κ receptors) able to interact preferentially with drugs such as ketocyclazocine or with peptides such as Arg^6-Phe^7-Met-E has been suggested (5, 6). However, the presence in brain of distinct κ receptors is still controversial (5). Despite the possible cross-reactivity of a compound for the three kinds of sites, the occurrence of a good correlation between the binding properties of a compound and its activity on MVD and GPI assays allowed use of the ratio of potency of these two organs as a standard with which to characterize its μ or δ specificity (7). The pharmacological relevance of these two receptors is strongly supported (a) by the lack of cross-tolerance between μ and δ ligands at both MVD and brain levels (8); (b) by the specific actions elicited by a μ agonist (analgesia or sedative effects) (4, 9, 10), or by a δ agonist (behavioral effects) (11); (c) by the good correlation between brain areas implicated in these specific pharmacological responses and localizations of μ and δ receptors (4); and (d) by the weak but significant structural differences between μ and δ receptors evidenced at the molecular level by selective protection experiments (12). The importance of these findings in the study of the role of enkephalins in the central nervous system and in therapeutic perspectives has induced an intensive search for specific ligands of these two receptors (9, 10).

Taking into account the rigid structures of opiates, several authors have attempted to find conformational similarities between enkephalins and opiates at the receptor site (see ref. 13 for a review). These analogies involve the L-tyrosine moiety, which could mimic the tyramine residue present in a large number of opiates, and the aromatic ring of Phe^4, which exists in some very potent analgesics. Likewise the importance of the Gly^3 residue and the configuration of the constituting amino acids were also evidenced (9, 10). However, none of these structural studies has taken into account the existence of receptor heterogeneity. Starting from pharmacological results obtained in both GPI and MVD assays and following a rational approach, we recently synthetized opioid peptides highly specific for μ (14) or δ receptors (15).

In the present work, an extensive structure-activity study of these peptides and enkephalins, including some NMR experiments and theoretical calculations, allowed (a) the definition of the structural components required for a specific interaction at the μ or δ receptor sites, (b) an accounting for the dual interaction of the endogenous enkephalins at the two binding sites, (c) an exploration of roughly the main structural differences in the μ and δ receptor subsites and the mechanism of the receptor recognition process and (d) an explanation for the loss of activity of the tetrapeptide Tyr-Gly-Gly-Phe as compared with Met-E and Leu-E. All of these results could permit rational synthesis of enzyme-resistant and highly specific opioid peptides.

MATERIALS AND METHODS

Chemicals. Met-E, Leu-E, and D-Ala^2-D-Leu^5-E[1] were obtained from Bachem (Bubendorf, Switzerland). All of the other peptides were synthetized in our laboratory by the liquid-phase method described for **16** (15).

^{1}H-NMR studies. ^{1}H-NMR spectra were recorded on a Brüker WH 270 spectrometer operating at 270 MHz in the FT mode and equipped with a decoupler unit and variable temperature accessories. Temperature was controlled at ±1°. Chemical shifts were measured from an internal reference (TMS) and were accurate to ±0.01 ppm. ^{1}H-NMR spectra were obtained by dissolution of the peptides in DMSOd$_6$ (100%) solutions (10^{-3} M). The lack of aggregation (through dimeric intermolecular head-to-tail interactions) for the zwitterionic form of Tyr-Gly-Gly-Phe in DMSOd$_6$ at high concentration (2×10^{-2} M) was controlled by sedimentation experiments using an optical detection method. After ultracentrifugation, weak bands corresponding to monomeric species were obtained for Tyr-Gly-Gly-Phe as well as for its methyl ester (hydrochloride form), which cannot form electrically stabilized dimers.

Energy calculations. A Monte Carlo sampling procedure including long-range interactions was used to obtain statistical averages over chain conformations of the tetrapeptide Tyr-X-Gly-Phe (X = Gly, L-Ala, and D-Ala). Van der Waals, H-bonded terms, electrostatic interactions, and torsional potentials were computed for energy calculations. All molecules are represented by their complete set of atoms with the exception of the alanine residue, whose C-methyl group is considered as a single composite atom. Generation of statistical samples of molecular chains were carried out as described previously (16). The constrained energy minimization was performed using a constraint function from the distance, in a least-squares sense, between selected atoms in the tetrapeptide **11** and morphine (16).

Pharmacological assays. Opioid activity was determined on the GPI and on the MVD as described previously (7, 15). Six different concentrations of each compound (6–10 assays for each) were tested for inhibition of electrically induced contractions. IC$_{50}$ values were computed by regression analysis. According to the method of Kosterlitz *et al.* (7), Met-E was used in each assay as the internal standard in order to avoid differences of sensitivity of each preparation, and the results obtained for the various derivatives were normalized using the relationship IC$_{50}$ (normalized) = IC$_{50}$ (computed value in the individual assay) × IC$_{50}$ (mean value of Met-E in the group of assays)/IC$_{50}$ (computed value of Met-E in the individual assay). By this method, the results obtained for Compounds **1–5**, **8**, and **17** are in good accord (±15%) with those already reported (7). In the GPI, K_e values were determined for morphine and peptide **11** from dose-response curves in the presence and absence of 20 nM

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[3] The abbreviations used are: MVD, mouse vas deferens; GPI, guinea pig ileum; Met-E, methionine-enkephalin; Leu-E, leucine-enkephalin; E, enkephalin; DMSOd$_6$, hexadeutero-dimethyl sulfoxide.

naloxone, as described by Kosterlitz and Watt (17). The reported values are the means ± standard error of the mean of three determinations in the presence and in the absence of naloxone for each compound.

Binding and competition experiments. [^{3}H]dihydromorphine and ^{3}H-labeled D-Ala^2-D-Leu^5-enkephalin were obtained from New England Nuclear Corporation (Boston, Mass.). ^{3}H-Labeled Tyr-D-Ser-Gly-Phe-Leu-Thr was synthesized at the Centre de l'Energie Atomique (Saclay) from its dibromotyrosyl precursor prepared in our laboratory. Binding studies on rats were performed as elsewhere described (2). Details of these experiments will be published elsewhere.[4]

RESULTS AND DISCUSSION

The μ or δ receptor specificity of the studied compounds was evaluated by comparison of their potency to inhibit the electrically evoked contractions of the GPI (μ receptors) and MVD (δ receptors). Starting from these experiments, the determination of structural requirements for selective interaction with μ or δ receptors necessitates that the following assumptions are satisfied: (a) pharmacological responses must be related to interactions with the same kind of receptors in both organs; (b) specificity determined from pharmacological assays must be well-correlated with the potency to inhibit the binding of labeled μ and δ agonists in brain homogenates; (c) κ receptors must not be significantly involved in the biological effects; and (d) susceptibility to degrading enzymes have to be similar. These assumptions were checked in this work for the peptides exhibiting the highest specificity: Tyr-D-Ser-Gly-Phe-Leu-Thr, **16** (δ binding sites), and Tyr-D-Ala-Gly-NH-CH(CH$_3$)-CH$_2$-CH(CH$_3$)$_2$, **11** (μ binding sites).

Structural Requirements for Specific Interaction at the μ-Receptor Site

The results in Table 1 show the well-known opposite effects of morphine, **1**, Met-E, **2**, and Leu-E, **3**, on the two isolated organs. Both endogenous peptides exhibit a higher potency in the MVD assay, and, as often stressed, Met-E is more potent than Leu-E on GPI; the reverse is true on MVD. The replacement of glycine in position 2 by D-alanine protects analogues **4** and **5** from degradation by aminopeptidases increasing their potency in the two assays (7). However, although the activity is enhanced only five or six times in both GPI and MVD for D-Ala^2-Met-E and in MVD for D-Ala^2-Leu-E, the latter is about 12 times more potent in the GPI assay than is Leu-E. Consequently, the D-Ala^2-substituted enkephalins **4** and **5** exhibit a similar potency on the GPI which contrasts with the differential activity of their parent compounds in the same test. This interesting feature, also reported by Kosterlitz *et al.* (7), could indicate that the discriminating role of the fifth amino acid of Met-E for its relative μ receptor specificity was eliminated by introduction of the D-Ala^2 residue. This implies that the methyl group of this D-amino acid could be specifically recognized by a μ receptor subsite. This assumption is strongly supported by the comparative potencies of two series of tetrapeptides containing the sequences Tyr-Gly-Gly-Phe, **6**, **7**, and Tyr-D-Ala-Gly-Phe, **8–10** (Table 1).

Evidence for a specific and hydrophobic binding subsite in μ receptor. In Tyr-Gly-Gly-Phe, **6**, the lack of a fifth amino acid severely decreases the potency on both receptors, although this compound remains a relatively good ligand for the enkephalin-binding sites (δ receptors), since its potency on MVD is only 2.5 times lower than that of morphine. This result is in good agreement with its better inhibition potency against ^{125}I-labeled D-Ala-D-Leu-E than against [^{3}H]dihydromorphine binding (2). The *N*-methylation of the Phe^4 residue as well as the esterification, amidification, or removal of the COOH-terminal carboxyl group do not increase significantly the potency of Tyr-Gly-Gly-Phe, as shown by the IC$_{50}$ values of these analogues, **7**, which are at least 100 times higher than those exhibited by Met-E (Table 1). The weak activity of Tyr-Gly-Gly-Phe and its derivatives strongly contrasts with the high potency of the corresponding tetrapeptides with a D-Ala^2 residue in place of Gly^2. In this series, **8–10**, the removal of the fifth amino acid leads to a high specificity for the μ receptors of GPI as shown by the ratio of inhibitory potencies on the two organs (18). The most efficient interaction of the D-Ala residue with the μ receptor is shown by the potency of **8**, which is a factor of 77 greater than that of **6** in the GPI but only 15 times higher in the MVD. Likewise, the activity of the pentapeptide Tyr-D-Ala-Gly-Phe-Leu is almost 10 times higher than its Gly^2 analogue on the GPI test (10). On the other hand, the gradual increase of activity following the elimination (Compound **9**) or the amidification (Compound **10**) of the charged COOH-terminal group could indicate an adverse interaction of this highly polar moiety within the μ receptor. Thus, the amidated tetrapeptide **10** is 8 times more potent than its parent compound on the GPI test.

Nevertheless, the highly decreased potency of Tyr-Gly-Gly-Phe and its derivatives could have been related either to an enhanced susceptibility to degrading enzymes or to a conformational constraint inhibiting the receptor recognition process. The first explanation can be rejected since (a) the rates of cleavage of the Tyr-Gly peptide bond by brain aminopeptidases are similar in Tyr-Gly-Gly-Phe and in Met-E or Leu-E; and (b) the lack of a fifth amino acid protects this peptide from the action of enkephalinase (19), a more specific enkephalin-degrading dipeptidyl carboxypeptidase.

On the other hand, Tyr-Gly-Gly-Phe exists in both solid and solvated states (20) under a highly folded conformation characterized by a head-to-tail interaction and a hydrogen bond between the CO of Tyr^1 and the NH of Phe^4. The restricted flexibility of this structure, easily evidenced in NMR by the shielding and the very small temperature dependence of the hydrogen-bonded NH of Phe^4 (Table 2), could inhibit the receptor interaction of Tyr-Gly-Gly-Phe. However, this assumption can also be ruled out, since the *N*-methylation of Phe^4 as well as the removal of the COOH group of **6** or its amidification do not increase the activity, although such modifications decrease the stability of the folded form as shown by the normal temperature dependence of the

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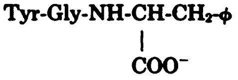
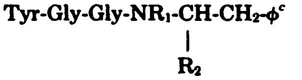
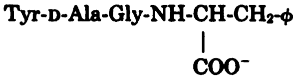
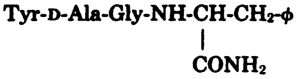
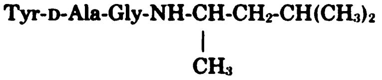
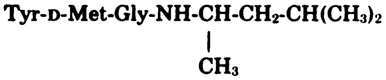
[4] M. David, J. C. Meunier, G. Gacel, J. L. Morgat, and B. P. Roques, submitted for publication.

TABLE 1

Inhibitory potencies of morphine and enkephalin derivatives on GPI and MVD assays and relative potencies to Met-E = 1

	Compound	GPI[a]		MVD[a]		$IC_{50}GPI/IC_{50}MVD$
		IC_{50}	Relative potency	IC_{50}(nM)	Relative potency	
		nM		*nM*		
1	Morphine	70 ± 8	2.86	390 ± 30	0.03	0.18
2	Tyr-Gly-Gly-Phe-Met	200 ± 19[b]	1.00	13 ± 1.5[b]	1.00	15.38
3	Tyr-Gly-Gly-Phe-Leu	393 ± 35	0.51	8.2 ± 1.6	1.59	47.93
4	Tyr-D-Ala-Gly-Phe-Met	35 ± 3	5.70	2.6 ± 0.5	5.00	13.46
5	Tyr-D-Ala-Gly-Phe-Leu	32 ± 3	6.25	2.1 ± 0.5	6.19	15.24
6	 $\text{Tyr-Gly-NH-}\underset{\underset{\text{COO}^-}{\vert}}{\text{CH}}\text{-CH}_2\text{-}\phi$	17,000 ± 500	0.012	1,000 ± 120	0.013	—
7	 $\text{Tyr-Gly-Gly-NR}_1\text{-}\underset{\underset{\text{R}_2}{\vert}}{\text{CH}}\text{-CH}_2\text{-}\phi^c$	≥15,000	≤0.015	≥1,000	≤0.01	—
8	 $\text{Tyr-D-Ala-Gly-NH-}\underset{\underset{\text{COO}^-}{\vert}}{\text{CH}}\text{-CH}_2\text{-}\phi$	220 ± 20	0.91	65 ± 5	0.20	3.40
9	Tyr-D-Ala-Gly-NH-CH_2-CH_2-ϕ	155 ± 35	1.30	325 ± 30	0.04	0.48
10	 $\text{Tyr-D-Ala-Gly-NH-}\underset{\underset{\text{CONH}_2}{\vert}}{\text{CH}}\text{-CH}_2\text{-}\phi$	25 ± 2	8.00	180 ± 20	0.07	0.14
11	 $\text{Tyr-D-Ala-Gly-NH-}\underset{\underset{\text{CH}_3}{\vert}}{\text{CH}}\text{-CH}_2\text{-CH(CH}_3)_2$	182 ± 21	1.10	1,220 ± 210	0.01	0.15
12	 $\text{Tyr-D-Met-Gly-NH-}\underset{\underset{\text{CH}_3}{\vert}}{\text{CH}}\text{-CH}_2\text{-CH(CH}_3)_2$	100 ± 12	2.00	1,100 ± 135	0.012	0.09
13	Tyr-D-Ala-$NHCH_2CH_2$-NH-CH_2-CH_2-ϕ	18,000 ± 500	0.011	>1,000	<0.01	—
14	Tyr-DAla-$NHCH_2CO$-NH_2	7,700 ± 500	0.026	>1,000	<0.01	—
15	Tyr-Gly-Gly-Phe-Met-Thr	380 ± 30	0.53	6.10 ± 2	2.13	62.3
16	Tyr-D-Ser-Gly-Phe-Leu-Thr	360 ± 30	0.55	0.58 ± 0.1	22.4	620
17	Tyr-D-Ala-Gly-Phe-D-Leu	41.2 ± 5	4.85	0.50 ± 0.09	24.1	82.4
18	Tyr-D-Ser-Gly-Phe-D-Leu	140 ± 20	1.43	0.85 ± 0.1	15.3	165
19	Tyr-D-Ser-Gly-Leu-Leu-Thr	2,000 ± 120	0.10	3,060 ± 400	0.004	—
20	Tyr-D-Ala-Gly-Phe-Leu-Thr	—		0.68[d]	19.12	
21	Tyr-D-Ala-Gly-Phe-D-Leu-Thr	—		17.1[d]	0.76	

[a] The values are the means ± standard error of the mean of 8–10 assays normalized using Met-E as the internal standard as described under Materials and Methods.

[b] Mean value corresponding to 182 assays in GPI and 207 assays in MVD.

[c] 7a: $R_1 = CH_3$, $R_2 = COOH$; 7b: $R_1 = R_2 = H$; 7c: $R_1 = H$, $R_2 = COOCH_3$; 7d: $R_1 = H$, $R_2 = CONH_2$.

[d] Computed from results of ref. 12.

Phe^4 amide proton in these compounds. Moreover, it is important to observe that Tyr-D-Ala-Gly-Phe exists in solution under a folded conformation (1–4 β-turn) similar to that found in the inactive Tyr-Gly-Gly-Phe (Table 2), pointing out that the presence of such kinds of folded forms in solution does not inhibit the μ receptor recognition process. Therefore, the decreased activity of Tyr-Gly-Gly-Phe, especially on the μ receptor, can be attributed to a loss of the required critical interactions at the level of this binding site since this compound does not exhibit antagonist properties. The preferential conformations determined in dimethyl sulfoxide may not necessarily be identical with those occurring in aqueous medium. However, as often stressed, the medium surrounding synaptic receptors buried in lipidic membranes is probably best mimicked by dimethyl sulfoxide than by water.

All of these results and those obtained in the pentapeptide series strongly support a specific interaction of the D-Ala^2 residue with an assumed hydrophobic subsite located in the μ receptors. This improved interaction, already proposed by Bajusz *et al.* (22), seems to involve the lateral chains of any hydrophobic aminoacids (10) and is highly stereospecific, since a change in the D-configuration of these critical residues causes an almost complete loss of activity in both the tetra- and pentapeptide series (9, 10).

δ-Inducing role of the aromatic ring of Phe^4. Although the presence of a D-Ala^2 residue and the shortening of the sequence of Met-E or Leu-E induce a strong preference for μ receptors, the potency on MVD of these tetrapeptides remains higher than or similar to that of morphine. A decisive shift towards μ receptor specificity is obtained in Compound **11** by the replacement of the phenyl ring of the decarboxy Tyr-D-Ala-Gly-Phe, **9**, by a hydrophobic alkyl chain. The single change between

TABLE 2

^{1}H-NMR parameters, computed coupling constants, and dihedral angles for Tyr-Gly-Gly-Phe, 6, and Tyr-D-Ala-Gly-Phe, 8

	Residue	^{3}J coupling constant			Dihedral angle		NH
		Exp.[a]	Calc.[b]	Calc.[c]	ϕ^d	ψ^d	dδ/dT × 10^3 ppm/°
6	Gly^2	4.8[e]	5.0	4.8	$-\underline{65}$°; $\underline{80}$°; ±180°	±$\underline{30}$°; ±150°	−12.0
	Gly^3	5.8[e]	5.8	6.3	±$\underline{65}$°; ±180°	±$\underline{30}$°; ±150°	−7.4
	Phe^4	8.0	8.1	7.3	−$\underline{80}$°; −160°	−$\underline{30}$°; +150°	0
8	D-Ala^2	7.0	6.1	7.2	+$\underline{80}$°; +160°	+$\underline{30}$°; +150°	−10.0
	Gly^3	5.7[e]	4.8	5.2	±$\underline{65}$°; ±180°	±$\underline{30}$°; ±150°	−10.0
	Phe^4	7.0	9.0	7.3	−$\underline{90}$°; −150°	−$\underline{30}$°; +150°	+4.0

[a] In Me_2SOd_6, 10^{-3} M, 20°.

[b] Averaged values from curves of Fig. 1.

[c] Obtained using the curves of Cung *et al.* (21) for the single conformation computed from Monte Carlo theoretical analysis (16), which gives the best agreement with experimental values. This particular conformation corresponds to an energy minimum (not higher than 0.5 Kcal/mole above the conformation of lowest energy).

[d] ϕ values were determined from the curves of Cung *et al.* (21), and ψ angles using conformational steric maps as described (20). The underlined values correspond to classical dihedral angles for β-turns.

[e] Mean value of two different coupling constants for non-equivalent glycine protons.

these two peptides (replacement of the phenethylamino chain of **9** by a 2,4-dimethylbutylamino chain in **11**) leads to a large decrease in δ receptor recognition. The ratio of inhibitory potencies (and therefore the μ specificity) is still enhanced by the replacement of D-Ala^2 by a D-Met^2 residue in **12** according to a probable adjustment of the lateral chain of methionine in the specific hydrophobic subsite of μ receptors. The high μ specificity of these short peptides strongly supports the proposed crucial role of the phenyl ring of Phe^4 in the differential interaction of enkephalins with μ and δ binding sites (14). Finally, the minimal requirements for specific binding to μ receptors are nicely evidenced by the loss of potency following the reduction of the CO of Gly^3 (Compound **13**) or the removal of the *N*-substituted hydrophobic chain (Compound **14**). This latter result could indicate that the interaction of an additional hydrophobic side-chain in a probably nonspecific subsite, which could correspond to the binding site F in the opiate receptor model of Feinberg *et al.* (23), might be required for activity. Nevertheless, the loss of potency of **14** could also be related to its decreased lipophilicity and therefore to unfavorable pharmacokinetic properties.

Conformational similarity of morphine and μ specific opioid peptides. Compounds **11** and **12**, obtained from this rational approach, exhibit a μ affinity similar to that of morphine (Table 1). This feature seems to indicate that, according to their small size and their high hydrophobic content, these short peptides are able to fit the conformational space occupied by a rigid and compact structure like morphine. This assumption was checked by conformational energy calculations performed on Tyr-X-Gly-Phe, with X = Gly, L-Ala (inactive compounds) and X = D-Ala (active peptide). The distribution curves of the distances between the C_α of tyrosine and C_α of phenylalanine, which can vary from 5–6 Å (highly folded conformation) to 10–12 Å (fully extended form) are reported in Fig. 1. The curves present a maximum corresponding to compact structures but with a higher folding tendency for Tyr-D-Ala-Gly-Phe. These results are supported by the correlation between the experimental ^{3}J coupling constants connected to the conformational states of the peptide backbone and their averaged values computed from the distribution curves of Fig. 1 (Table 2). Although the statistical sample is a good model for **6**, the behavior of **8** can be better explained by the existence of a well-defined conformation as shown by the agreement between its ^{3}J experimental values and those computed for the lowest-energy conformer with a strong head-to-tail interaction.

Starting from the folding tendency of μ-specific peptides and the absolute requirement for activity of both a tyrosine moiety and an intact Gly^3 CO group, we have recently proposed a constrained energy minimization procedure (16) that enables demonstration of the spatial correspondence of the main biologically required functional groups in morphine and tetrapeptides. Figure 2 shows the great analogy between the classifical T-shape of morphine and the computed conformation of **11**. This structure is characterized by (a) an energetically stabilized folded form only 4–5 Kcal/mole higher than the lowest nonconstrained conformer (such a small loss of

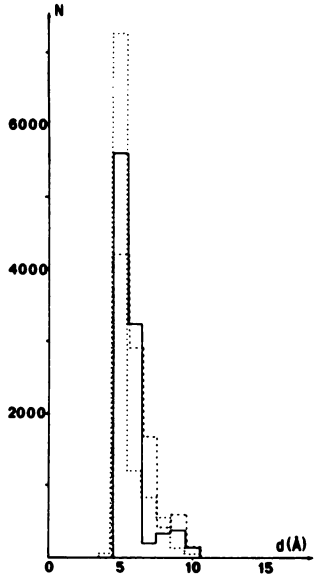


FIG. 1. *Distribution curves of the $C_1^\alpha \ldots C_4^\alpha$ distance in H_3N^+-Tyr-X-Gly-Phe-COO$^-$ tetrapeptides*

—— X = Gly; ----- X = L-Ala; ···· X = D-Ala.

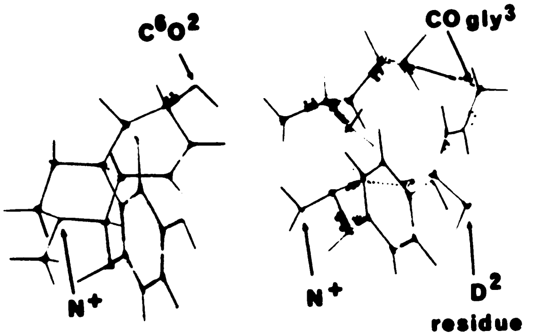


FIG. 2. *Molecular models of the proposed biologically active peptide and of morphine*

Right. Molecular model of the proposed biologically active conformation of Tyr-D-Ala-Gly-NH-$\underset{\substack{| \\ CH_3}}{CH}$-$CH_2$-$CH(CH_3)_2$ at the μ receptor site. In this T-shaped conformer of low energy the computed backbone angles (IUPAC) are as follows: $\psi_1 = -173°$, $\phi_2 = 99°$, $\psi_2 = 41°$, $\phi_3 = 142°$, $\psi_3 = -113°$, $\phi_4 = 10°$. The spatial disposition of the tyramine moiety corresponds to that found in morphine, and the CO of Gly^3 corresponds to the C_6O_2 group of the alkaloid. This conformation reveals the lateral disposition of the side-chain (CH_3 in this case) of a D^2-amino acid.

Left. Molecular model of morphine.

energy is probably easily counterbalanced by the large free energy changes through hydrophobic, hydrogen-bonded, and electrostatic interactions within the receptor site); (b) an external orientation of the lateral chain of any D-amino acid in position 2, allowing its additional binding to a hydrophobic area located in the μ receptor site; (c) a good correspondence between the tyrosine moiety of **11** and the phenethylamine chain of morphine; and (d) a similar spatial orientation of the C_6—OH group of morphine and the CO of Gly^3. The importance for pharmacological activity of an oxygen atom located in the C_6–C_8 area of morphine has already been emphasized (3).

These proposed μ-specific requirements and the conformational parameters of the computed conformation reported in the legend to Fig. 2 agree (16) with (a) the potency of highly constrained enkephalins containing an α-amino isobutyric residue in position 2 or a double bond at the level of the Phe^4 residue and with the retention of activity following the reduction or replacement by an ethylenic bond of the Tyr^1-Gly^2 or Gly^2-Gly^3 amide groups; (b) the correlation between the folding tendency of a series of D-X^2-Pro^5 enkephalins and their μ specificity; and (c) the low μ potency of Tyr-Gly-Gly-Phe-Y (Y = Gly, Ala, Pro, Thr, or Ser) (9, 10) in which the absence of a D-amino acid in position 2 could not be counterbalanced by the interaction of too-short (Gly, Ala), sterically hindered (Pro), or hydrophilic (Thr, Ser) side-chains of the fifth amino acid with the assumed hydrophobic subsite. In contrast, this interaction probably occurs in the case of Met-E, Leu-E, or Tyr-Gly-Gly-Phe-Y (Y = Ile, Nle), since all of these peptides are good μ agonists (10).

Structural Requirements for Specific Interaction at the δ Receptor Site

In the first part of this work we have shown that a peptide can mimic the rigid structure of opioid alkaloids. Obviously, an even more interesting aim was to increase strongly the δ specificity of enkephalins. For such a purpose the following features were taken into account: (a) the phenyl ring of Phe^4 seems to be required for δ receptors recognition (2, 14); (b) a Leu^5 amino acid induces a higher δ specificity than does a Met^5 residue (Table I); and (c) addition of a hydrophilic moiety (Thr^6) to the enkephalin sequence strongly increases the δ specificity (9) as shown by the ratio of the IC_{50} values of **15** on the two organs (Table 1).

Moreover, since hydrophobic D-amino acids instead of Gly^2 enhance the μ specificity, a hydrophilic residue introduced in position 2 was expected to decrease this μ-improving factor. As shown in Table 1, the lengthening of the Leu-E sequence by a Thr^6 residue and the replacement of Gly^2 by D-Ser^2 led to the hexapeptide Tyr-D-Ser-Gly-Phe-Leu-Thr, **16**, which exhibits a 620-times higher potency on MVD than on GPI. This compound is thus about 10 times more specific for peripheral δ receptors than is D-Ala^2-D-Leu^5-E, **17**, a compound commonly used to characterize the δ receptors in binding or pharmacological experiments (2, 4, 7, 8, 11). The importance of the hydrophobic subsite located in μ receptors is again clearly demonstrated by the significant increase towards δ specificity following the replacement of the D-Ala^2 residue by a D-Ser^2 amino acid in **18**.

Finally, as expected, the phenyl ring of Phe^4 seems to play a crucial role in δ receptor recognition since its replacement by leucine, which is a large and hydrophobic amino acid, leads to a complete loss of activity on MVD but only a 6-times lower potency on GPI (Compound **19**).

It can be observed that, in several enkephalin analogues containing a D-aminoacid in position 2, the replacement of the Phe^4 residue by the hexahydrophenylalanine residue leads to a larger decreased activity on the MVD than on the GPI assays (24). This result could suggest that the aromatic character of the fourth residue is important for δ receptor interaction.

Another striking result concerns the relationship between the δ specificity and the stereochemistry of the fifth amino acid. Indeed, as shown in Table 1, the replacement of Leu^5 by its stereoisomer leads to a high preference for the δ receptors in pentapeptides **5** and **17** . However, as already stressed by Bedell *et al.* (9), this stereospecific dependence of δ specificity is entirely reversed by the addition of a Thr^6 residue, since hexapeptide **21** exhibits a 25-times lower activity than its analogue **20** with an L-Leu in position 5.

These large differences probably reflect the ability of the Phe^4 side-chain to fit into its δ-specific subsite, which depends on the conformational constraint raised by the following amino acids. This assumption is supported by a recent conformational analysis (25) revealing the very large difference in the Phe^4 side-chain orientation in enkephalins bearing an L- or a D-Leu residue in the fifth position. Thus a D-Leu^5 amino acid leads to a unique and roughly extended conformation of the peptide with the D-Leu^5 side-chain near the phenyl ring of Phe^4. In contrast, the enkephalins with an L-Leu^5 residue exist in equilibrium between folded and extended forms with the leucine side-chain directed toward the tyrosine ring in the folded conformer. This result is in good accord with the folding requirement for μ receptor interaction and therefore with the dual interaction of Leu-E with the two binding sites. Moreover, NMR experiments confirm this theoretical conformational analysis, since in the δ-specific peptides **17** and **18** the protons of the D-Leu side-chain are upfield-shifted as compared with their position in **5** (not shown). This shielding is probably related to a proximity between the D-Leu side-chain and the Phe^4 aromatic ring. This assumption is supported by the similar behavior of the L-Leu side-chain protons in the highly δ specific hexapeptide **16**. Finally, the increased population of extended forms in **16** is evidenced by the higher temperature dependence of all of the peptide protons ($d\delta/dT > 3 \times 10^{-3}$ ppm/°).

Therefore, it appears that the preferential recognition of δ binding sites requires (a) a phenyl ring in position 4; (b) a conformational orientation of the COOH-terminal part of the peptide, allowing fitting of the Phe^4 side-chain in a δ-specific subsite; and (c) a more extended conformation of the peptide. Furthermore, a high hydrophobic content, especially at the level of the second amino acid, decreases the δ-receptor recognition and then enhances the μ receptor specificity.

Synthetic opiates such as etorphine and several analogues in the oripavine series which exhibit similar affinities for μ and δ binding sites (2, 3) also satisfy such requirements. Indeed, these derivatives have a larger size than the classical opiates and contain an alkyl or aromatic chain which could interact with a hydrophobic area of the receptor and a hydroxyl group which could mimic that of the Thr^6 in **16**. According to the presence in its structure of all of the critical components required to interact with both μ and δ receptors, the very potent peptide Tyr-D-Ala-Gly-MePhe-Met(O)-ol FK 33824 exhibits a high potency on GPI and MVD assays and inhibits equally well opiate or peptide binding in brain homogenates (7).

Extent of Structure-Activity Relationships Performed on Isolated Organs to Central Nervous System: Usefulness and Limits

As described at the beginning of this work, the validity of the proposed structural requirements for specific receptor recognition requires that several assumptions must be satisfied. The first assumption concerns the interaction of the most selective peptides to a single kind of receptor. This can be established by a comparison of the relative potencies of the μ and δ-specific peptides to morphine in both GPI and MVD assays (26). Thus, the respective ratios of activity (morphine = 1) on GPI and MVD are 0.46 and 1.20 for **9**; 2.8 and 2.0 for **10**; 0.38 and 0.32 for **11**; 0.10 and 0.35 for **12**; 0.18 and 672 for **16**; 1.70 and 780 for **17**; and 0.50 and 458 for **18**. These features strongly suggest that the short peptides **9, 10, 11, 12**, and morphine interact in both organs with the same kinds of receptors, μ. (26). In contrast, the much higher values of the ratios on MVD (rich in enkephalin receptors) than on GPI assays agree with the pharmacological activities of **16, 17**, and **18**, through δ receptor stimulation. Moreover, the lack of interaction of the μ-specific peptide **11** with κ receptors which could be present in GPI (27) is evidenced by the similar antagonist potency of naloxone against morphine (K_e = 2.1 ± 0.18 nM) or against **11** (K_e = 3.6 ± 0.4 nM) in the GPI assay. Taken together, all of these results show that μ or δ-specific peptides interact chiefly with their own kinds of receptors in the two isolated organs (26). The relevance of this structural approach at the level of brain receptors was checked with the specific peptides **11** and **16**. This latter compound was tritiated using its dibromotyrosyl analogue. The binding experiments performed on rat brain homogenates at 35° in Tris-HCl buffer (pH 7.4) show that between 0.2 and 20 nM ^{3}H-labeled Tyr-D-Ser-Gly-Phe-Leu-Thr interacts with one single class of binding sites (K_D = 2.7 nM; capacity = 0.11 pmole/mg).^{4} Under the same conditions, the tritiated peptide **17**, ^{3}H-labeled Tyr-D-Ala-Gly-Phe-D-Leu, binds with two classes of sites (K_{D_1} = 2.0 nM; c = 0.10 pmole/mg; K_{D_2} = 20 nM; c = 0.20 pmole/mg) in accordance with the results of a previous study performed with ^{125}I-labeled Tyr-D-Ala-Gly-Phe-D-Leu (2). Therefore, as expected, the pharmacological profiles of **16** and **17** are in accord with their potencies on isolated organs, but, in addition, **16** behaves as a pure δ agonist in a large concentration range. Likewise, the selectivity of **11** for μ receptors of rat brain was clearly established, since this compound is almost 50 times more potent in competing for the binding of [^{3}H]dihydromorphine (μ receptors) (IC$_{50}$ ~ 20 nM) than for ^{3}H-labeled Tyr-D-Ala-Gly-Phe-D-Leu (δ receptors) (IC$_{50}$ ~ 1000 nM).

These experiments were performed in the absence of added sodium or GTP in order to avoid a change in binding affinities with the two kinds of receptors (2). It can be noted that, in contrast, κ agonists usually exhibit comparable affinity with both μ and δ binding sites (5).

The possibility that the selectivity of these peptides is related to their differential susceptibilities to degrading enzymes could not be completely excluded. However, this hypothesis is very unlikely since, as already shown (15), hexapeptide **16** remains unchanged after 1 hr of incubation with rat brain membranes. Moreover, the presence of a D-amino acid in position 2 and the shortening (for μ peptides) or the lengthening (for δ peptides) of the sequences of natural enkephalins strongly inhibit their degradation by both aminopeptidase and enkephalinase activities (19). Therefore, the large differences in selectivity or potency between compounds such as **9** and **11** or **16** and **19** are more probably related to their structural differences at the level of receptor interactions.

CONCLUSION

The results of this study suggest that, according to selective protection experiments (12), subtle changes exist in the subsites of μ and δ receptors. Obviously, at this time a precise mapping of both receptors or information on their chemical constituents cannot be obtained. However, it appears that μ receptors bind preferentially highly hydrophobic compounds with compact structures, such as the recently described morphiceptin (28), whereas δ receptors interact more strongly with longer peptides which can be more hydrophilic. Taking into account the good relationship between the lipophilicity of classic opiates and their analgesic potencies (29), the presence of lipids as critical components of the μ binding site cannot be rejected. These differences in the size of the two receptors and the position of the assumed specific binding subsites illustrated in Fig. 3 allow (a) synthesis

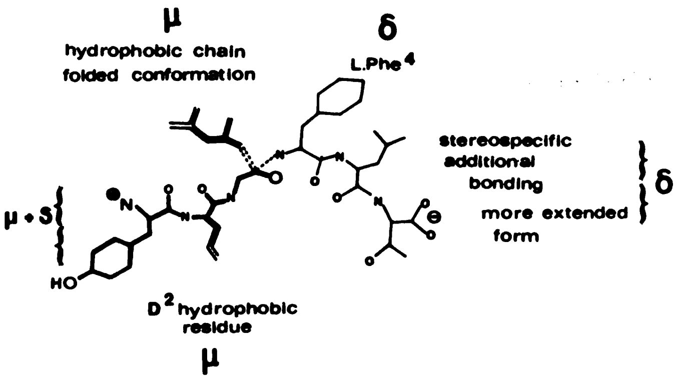


FIG. 3. *Scheme of the main structural requirements for specific interactions with μ or δ opiate receptors*

of peptides which discriminate between the two receptors and (b) an explanation of the dual activity of endogenous enkephalins by adjustment of the side-chains of their fourth and fifth amino acids in the appropriate subsites of δ or μ receptors and therefore the decreased potency of Tyr-Gly-Gly-Phe. The differences between the conformation of a μ-specific peptide in solution (deduced from NMR) and at the receptor level (proposed from computed similarity with morphine) suggest a stepwise mechanism for the receptor recognition process. The biological relevance of these findings at the level of brain receptors is strongly supported by binding experiments on rat brain homogenates.

Therefore, compounds such as **11** and **16** could be used to study the distribution and the physiological role of the two kinds of receptors. Thus, we have recently shown that **11** and **16**, which have similar potencies in the GPI assay (μ receptors) but differ by approximately three orders of magnitude in activity on the MVD (δ receptors) exhibit similar analgesic properties in rats (30). These features strongly support the proposed preferential involvement of μ receptors in antinociceptive responses (4, 7, 9, 10). The very interesting behavioral actions conveyed by brain δ receptors (11) can be henceforth explored by using such δ-specific ligands.

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REFERENCES

1. Lord, J. A. H., A. A. Waterfield, J. Hughes, and H. W. Kosterlitz. Endogenous opioid peptides: multiple agonists and receptors. *Nature (Lond.)* **267**:495–499 (1977).
2. Chang, K. J., and P. Cuatrecasas. Multiple opiate receptors: enkephalins and morphine bind to receptors of different specificity. *J. Biol. Chem.* **254**:2610–2618 (1979).
3. Childers, S. R., I. Creese, A. M. Snowman, and S. H. Snyder. Opiate receptor binding affected differentially by opiates and opioid peptides. *Eur. J. Pharmacol.* **55**:11-18 (1979).
4. Goodman, R. R., S. H. Snyder, M. J. Kuhar, and W. S. Young III. Differentiation of delta and mu opiate receptor localizations by light microscopic autoradiography. *Proc. Natl. Acad. Sci. U. S. A.* **77**:6239–6243 (1980).
5. Chang, K. J., E. Hazum, and P. Cuatrecasas. Possible role of distinct morphine and enkephalin receptors in mediating actions of benzomorphan drugs (putative κ and σ agonists). *Proc. Natl. Acad. Sci. U. S. A.* **77**:4469–4473 (1980).
6. Audigier, Y., H. Mazarguil, J. Rossier, and J. Cros. Met-enkephalin $(\text{Arg}^6,\text{Phe}^7)$ interacts with the κ-receptors on guinea pig ileum. *Eur. J. Pharmacol.* **68**:237–238 (1980).
7. Kosterlitz, H. W., J. A. H. Lord, S. J. Paterson, and A. Waterfield. Effects of changes in the structure of enkephalins and of narcotic analgesic drugs on their interactions with μ and δ receptors. *Br. J. Pharmacol.* **68**:333–342 (1980).
8. Schulz, R., M. Wuster, H. Krenss, and A. Herz. Selective development of tolerance without dependence in multiple opiate receptors of mouse vas deferens. *Nature (Lond.)* **285**:242-243 (1980).
9. Bedell, C. R., R. B. Clark, R. L. Follenfant, L. A. Lowe, F. B. Ubatuba, S. Wilkinson, and R. J. Miller. Analogues of the enkephalins: structural requirements for opioid activity. *Pharmacochem. Libr.* **2**:177-193 (1977).
10. Morley, J. S. Structure-activity relationships of enkephalin-like peptides. *Annu. Rev. Pharmacol. Toxicol.* **20**:81-110 (1980).
11. Stein, L., and J. D. Belluzzi. Brain endorphins and the sense of well-being: a psychobiological hypothesis. *Adv. Biochem. Psychopharmacol.* **18**:299–311 (1978).
12. Smith, J. R., and E. J. Simon. Selective protection of stereospecific enkephalin and opiate binding against inactivation by *N*-ethylmaleimide: evidence for two classes of opiate receptors. *Proc. Natl. Acad. Sci. U. S. A.* **77**:281–284 (1980).
13. Gorin, F. A., T. M. Balasubramanian, C. D. Barry, and G. R. Marshall. Elucidation of the receptor-bound conformation of the enkephalins. *J. Supramol. Struct.* **9**:27–39 (1978).
14. Roques, B. P., G. Gacel, M. C. Fournie-Zaluski, B. Senault, and J. M. Lecomte. Demonstration of the crucial role of the phenylalanine moiety in enkephalin analogues for differential recognition of the μ and δ receptors. *Eur. J. Pharmacol.* **60**:109–110 (1979).
15. Gacel, G., M. C. Fournie-Zaluski, and B. P. Roques. Tyr-D-Ser-Gly-Phe-Leu-Thr, a highly preferential ligand for delta-opiate receptors. *F. E. B. S. Lett.* **118**:245–247 (1980).
16. Maigret, B., S. Premilat, M. C. Fournié-Zaluski, and B. P. Roques. Proposals for conformation of enkephalins related to opiate mu-pharmacophore. *Biochem. Biophys. Res. Commun.* **99**:267–274 (1981).
17. Kosterlitz, H. W., and A. J. Watt. Kinetic parameters of narcotic agonists and antagonists with particular reference to naloxone. *Br. J. Pharmacol.* **33**:266–271 (1968).
18. Morgan, B. A., J. D. Bower, K. P. Guest, B. K. Handa, G. Metcalf, and C. F. C. Smith. Structure-activity relationships of enkephalin analogs, in *Proceedings of the Fifth American Peptide Symposium* (H. Godman and J. Meienhofer, eds.). J. Wiley and Sons, New York, 111–113 (1977).
19. Fournié-Zaluski, M. C., R. Perdrisot, G. Gacel, J. P. Swerts, B. P. Roques, and J. C. Schwartz. Inhibitory potency of various peptides on enkephalinase activity from mouse striatum. *Biochem. Biophys. Res. Commun.* **91**:130–135 (1979).
20. Fournié-Zaluski, M. C., T. Prange, C. Pascard, and B. P. Roques. Enkephalin related fragments, conformational studies of the tetrapeptides Tyr-Gly-Gly-Phe and Gly-Gly-Phe-X (X = Leu, Met) by X-ray and ^{1}H NMR spectroscopy. *Biochem. Biophys. Res. Commun.* **79**:1199–1206 (1977).
21. Cung, M. T., M. Marraud, and J. Neel. Experimental calibration of a Karplus relationship in order to study the conformations of peptides by nuclear magnetic resonance. *Macromolecules* **7**:606–613 (1974).
22. Bajusz, S., A. Z. Ronai, J. I. Szekely, Z. Dunai-Kovacs, I. Berzetei, and L. Graf. Enkephalin analogs with enhanced opiate activity. *Acta Biochim. Biophys. Acad. Sci. Hung.* **11**:305–309 (1976).
23. Feinberg, A. P., I. Creese, and S. H. Snyder. The opiate receptor: a model explaining structure-activity relationships of opiate agonists and antagonists. *Proc. Natl. Acad. Sci. U. S. A.* **73**:4215–4219 (1976).
24. Audigier, Y., H. Mazarguil, R. Gout, and J. Cros. Structure-activity relationships of enkephalin analogs at opiate and enkephalin receptors: correlation with analgesia. *Eur. J. Pharmacol.* **63**:35–46 (1980).
25. Manavalan, P., and F. A. Momany. A comparison of low energy structures of enkephalin analogs, in *Proceedings of the Sixth American Peptide Symposium* (E. Gross and J. Meienhofer, eds.). Pierce Chemical Company, Rockford, Ill., 893–896 (1979).
26. Handa, B. K., A. C. Lane, J. A. H. Lord, B. A. Morgan, M. J. Rance, and C. F. C. Smith. Analogues of β-LPH$_{61-64}$ possessing selective agonist activity at μ-opiate receptors. Eur. J. Pharmacol. **70**:531-540 (1981).
27. Hutchison, M., H. W. Kosterlitz, F. M. Leslie, A. A. Waterfield, and L. Terenius. Assessment in the guinea pig ileum and mouse vas deferens of benzomorphans which have strong antinociceptive activity but do not substitute for morphine in the dependent monkey. *Br. J. Pharmacol.* **55**:541–546 (1975).
28. Chang, K. J., A. Killian, E. Hazum, P. Cuatrecasas and J. K. Chang. Morphiceptin (NH_2-Tyr-Pro-Phe-Pro-$CONH_2$): a potent and specific agonist for morphine (μ) receptors. *Science (Washington. D. C.)* **212**:75–77 (1981).
29. Cho, T. M., J. S. Cho, and H. H. Loh. Physicochemical basis of opiate-cerebroside sulfate interaction and its application to receptor theory. *Mol. Pharmacol.* **16**:393–405 (1979).
30. Gacel, G., M. C.. Fournie-Zaluski, E. Fellion, and B. P. Roques. Evidence of the preferential involvement of mu-receptors in analgesia using enkephalins highly selective for peripheric mu or delta receptors. *J. Med. Chem.*, in press.

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