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The μ- and δ-opioid pharmacophore conformations of cyclic β-casomorphin analogues indicate docking of the Phe^3 residue to different domains of the opioid receptors*

Wolfgang Brandt**, Matthias Stoldt and Heiko Schinke

Institute of Biochemistry, Martin-Luther-University Halle-Wittenberg, Kurt-Mothes-Strasse 3, D-06099 Halle, Germany

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Summary

Cyclic β-casomorphin analogues with a D-configured amino acid residue in position 2, such as Tyr-c[-Xaa-Phe-Pro-Gly-] and Tyr-c[-Xaa-Phe-D-Pro-Gly-] (Xaa = D-A_2bu, D-Orn, D-Lys) were found to bind to the μ-opioid receptor as well as to the δ-opioid receptor, whereas the corresponding L-Xaa^2 derivatives are nearly inactive at both. Low-energy conformers of both active and nearly inactive derivatives have been determined in a systematic conformational search or by molecular dynamics simulations using the TRIPOS force field. The obtained conformations were compared with regard to a model for μ-selective opiates developed by Brandt et al. [Drug Des. Discov., 10 (1993) 257]. Superpositions as well as electrostatic, lipophilic and hydrogen bonding similarities with the δ-opioid receptor pharmacophore conformation of t-Hpp-JOM-13 proposed by Mosberg et al. [J. Med. Chem., 37 (1994) 4371, 4384] were used to establish the probable δ-pharmacophoric cyclic β-casomorphin conformations. These conformations were also compared with a δ-opioid agonist (SNC 80) and the highly potent antagonist naltrindole. These investigations led to a prediction of the μ- and δ-pharmacophore structures for the cyclic β-casomorphins. Interestingly, for the inactive compounds such conformations could not be detected. The comparison between the μ- and δ-pharmacophore conformations of the cyclic β-casomorphins demonstrates not only differences in spatial orientation of both aromatic groups, but also in the backbone conformations of the ring part. In particular, the differences in Φ^2 and Ψ^2 ($\mu \approx 70°,-80°$; $\delta \approx 165°,55°$) cause a completely different spatial arrangement of the cyclized peptide rings when all compounds are matched with regard to maximal spatial overlap of the tyrosine residue. Assuming that both the μ- and δ-pharmacophore conformations bind with the tyrosine residue in a similar orientation at the same transmembrane domain X of their receptors, the side chain of Phe^3 as a second binding site has to dock with different domains.

Introduction

Since Brantl and Teschemacher [1,2] discovered that fragments of the bovine β-casein peptone exhibit analgesic potential, a number of β-casomorphin analogues (β-casomorphin-5 = Tyr-Pro-Phe-Pro-Gly) have been synthesized and tested with regard to their μ- and δ-receptor binding affinity [3–9]. In particular, cyclic peptides such as Tyr-c[-Xaa-Phe-Pro-Gly-] and Tyr-c[-Xaa-Phe-D-Pro-Gly] (Xaa = D-A_2bu, D-Orn, D-Lys) were shown to have rather high antinociceptive activity in rats [7–9]. Interestingly, the corresponding ornithine compounds with an L-amino acid residue in position 2 show no or only very low activity at both opioid receptors (Table 1). The structure(conformation)–activity relationships of the ornithine-containing β-casomorphin derivatives have already been described with respect to their very likely μ-selective pharmacophoric conformations. The molecular modelling investigations were supported by NMR experiments [9–12].

Although the cyclic β-casomorphin analogues con-

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**To whom correspondence should be addressed.

TABLE 1
BINDING, GPI AND MVD ASSAYS OF CASOMORPHIN DERIVATIVES[a]

No.	Analogue	IC_{50} (nM)		IC_{50} ratio DADLE/DAGO	IC_{50} (nM)		IC_{50} ratio MVD/GPI
		[^{3}H]DAGO	[^{3}H]DADLE		GPI	MVD	
1	Tyr-c[-D-A_2bu-Phe-D-Pro-Gly-]	0.3	4.78	15.9	1.06	4.67	4.41
2	Tyr-c[-D-A_2bu-Phe-Pro-Gly-]	nd	nd	nd	nd	nd	nd
3	Tyr-c[-D-Orn-Phe-D-Pro-Gly-]	25.7	123	4.79	2.14	4.89	2.29
4	Tyr-c[-D-Orn-Phe-Pro-Gly-]	1.2	12	10	13.4	69.9	5.22
5	Tyr-c[-D-Lys-Phe-D-Pro-Gly-]	0.2	9.6	48	4.3	16.3	3.79
6	Tyr-c[-D-Lys-Phe-Pro-Gly-]	13.6	43	3.16	4.65	51.4	11.1
7	Tyr-c[-A_2bu-Phe-D-Pro-Gly-]	nd	nd	nd	nd	nd	nd
8	Tyr-c[-A_2bu-Phe-Pro-Gly-]	nd	nd	nd	nd	nd	nd
9	Tyr-c[-Orn-Phe-D-Pro-Gly-]	3 000	> 100 000	–	61 000	> 40 000	> 0.656
10	Tyr-c[-Orn-Phe-Pro-Gly-]	80 000	> 10 000	–	32 800	> 10 000	> 0.305
11	Tyr-c[-Lys-Phe-D-Pro-Gly-]	nd	nd	nd	nd	nd	nd
12	Tyr-c[-Lys-Phe-Pro-Gly-]	nd	nd	nd	nd	nd	nd

[^{3}H]DAGO = H-Tyr-D-Ala-Gly-MePhe-Gly-ol; [^{3}H]DADLE = H-Tyr-D-Ala-Gly-Phe-D-Leu-OH; GPI = guinea pig ileum; MVD = mouse vas deference; nd = not determined; A_2bu = diaminobutyric acid.
[a] Taken from Ref. 7.

sidered prefer to bind to the μ-receptor, their binding affinity to δ is not neglectably low (see Table 1). The different pharmacophoric conformations of the same compound responsible for binding to the μ- as well as to the δ-opioid receptor have never been discussed for β-casomorphin analogues. In order to synthesize compounds that are more selective to either receptor, such a comparison should be of high interest.

Several models have been described for the characterization of μ- and δ-pharmacophoric conformations [e.g. 13–24]. Not all of these studies led to clear descriptions of the three-dimensional structure of the bioactive conformations. Either the compounds taken into account showed high conformational flexibiliy or the theoretical methods used were not appropriate to reflect all conformational features well. One of the most rigid compounds displaying high δ-opioid receptor affinity is t-Hpp-JOM-13 (*trans*-3-(4'-hydroxyphenyl)Pro-c[-D-Cys-Phe-D-Pen-], where Pen is β,β-dimethyl-Cys). Mosberg et al. [18,19] used this compound to propose two δ-opioid receptor pharmacophoric conformations. These conformations were taken as target conformations for the analysis of the bioactive conformation of the cyclic β-casomorphins that will probably bind to the δ-opioid receptor. Furthermore, for comparison we included the nonpeptide δ-opioid receptor agonist SNC 80 [20] as well as the rigid and highly potent naltrexone-derived δ-opioid antagonist naltrindole (NTI) [21].

For the analysis of the μ-pharmacophoric conformations of the cyclic β-casomorphins, we used the procedure that was already successfully applied to cyclic D-Orn^2-β-casomorphin analogues [10]. Herein, all obtained conformations were compared with regard to spatial, electrostatic and hydrogen bonding similarities with the μ-pharmacophore conformation of PEO (7-α-[1-hydroxy-1-methyl-3-phenylpropyl]-6,14-*endo*-ethenotetra-hydrooripavine) [22]. The comparison led to suggestions for the bioactive conformations and, additionally, to explanations of the inactivity of L-Orn^2-β-casomorphin analogues [10]. Low-energy conformers of both active and nearly inactive cyclic β-casomorphins have been determined in a systematical conformational search or by molecular dynamics simulations using the TRIPOS force field [25].

Computational methods

Conformational analyses were performed with the molecular modelling program SYBYL 6.0 [26] running on SGI Crimson VGXT and IBM RISC/6000 workstations. Each cyclic A_2bu^2 (= diaminobutyric acid) β-casomorphin analogue was subjected to an extensive conformational search. Thus, in a first step we excluded the tyrosine residue and the phenylalanine side chain and performed a separate ring search for both a *trans*- and *cis*-proline peptide bond (bond length variance 0.3 Å, bond angle variance 15°). The exocyclic tyrosine residue and the phenylalanine side chain were added to all minimized low-energy ring conformations and a conformational search was subsequently performed by systematically rotating each rotatable bond in 30° increments. In the case of the Lys^2 analogues, we performed molecular dynamics simulations at 1000 K over 100 ps for the complete molecules for each *trans*- and *cis*-Pro^4 conformation separately. The conformations were collected every 100 fs and subsequently minimized until the energy change per step was less than 0.0001 kcal/mol. All conformations obtained from the searches were grouped into families, based on similarity of their dihedral angles (±30°). Using the TRIPOS force field [25] and the Powell minimizer contained in the SYBYL/MAXIMIN software package, the lowest energy member of each conformational family was then extensively minimized. The Gasteiger method was used to calculate the partial charge distribution of the

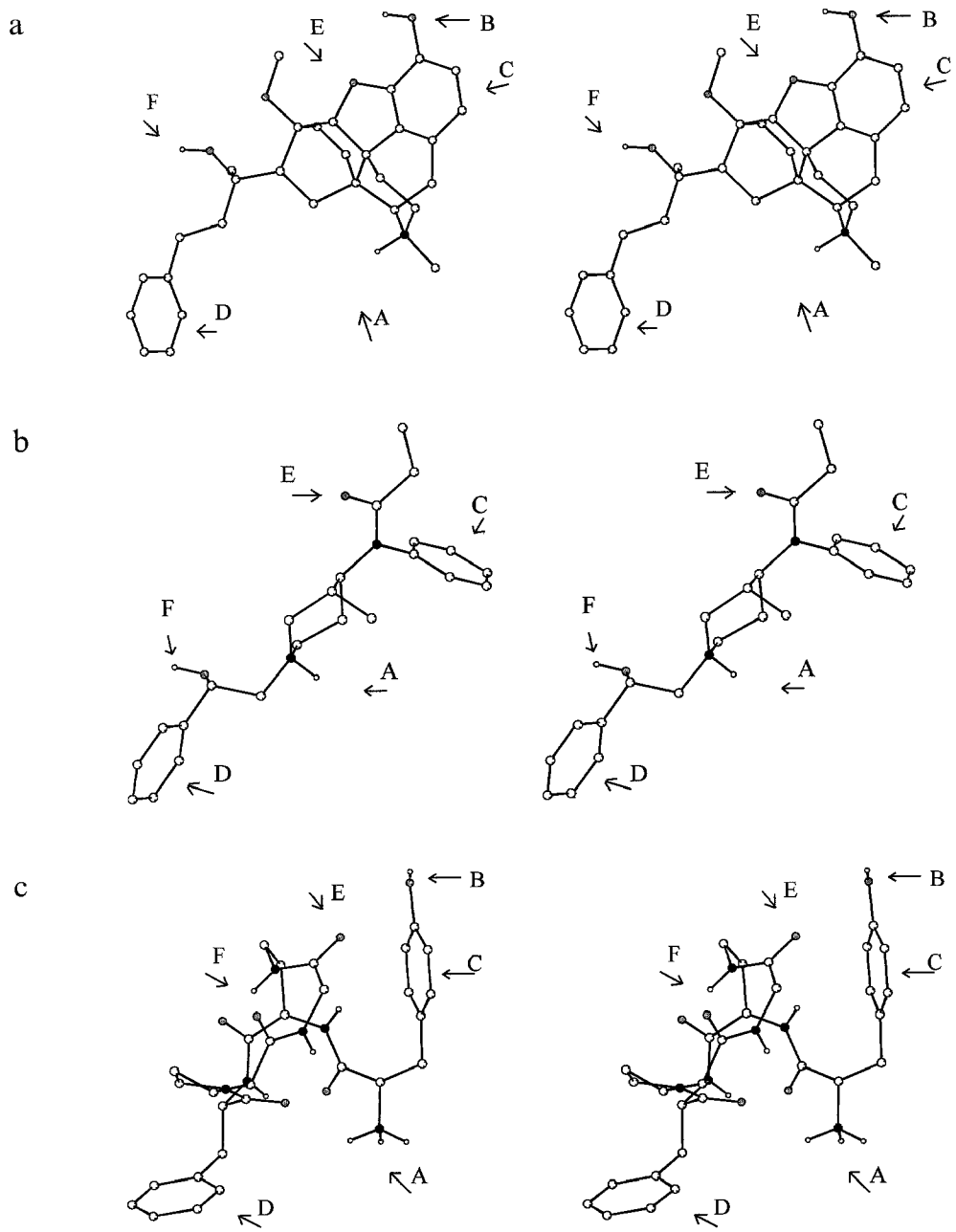

Fig. 1. Stereo representations of the proposed μ-pharmacophore conformations of (a) PEO; (b) *cis*-(3*R*,4*S*,2'*S*)-ohmefentanyl; and (c) Tyr-c[-D-A_2bu-Phe-D-Pro-Gly-]. The areas A to F indicate suggested essential binding sites to the μ-opioid receptor.

molecules (PEOE) [27,28]. Electrostatic interactions were taken into consideration by using a distance-dependent dielectric constant of $\varepsilon = 4$. All molecules were investigated with the N-terminal amino groups in the protonated form. An energy cutoff of 7 kcal/mol above the global energy minimum was applied to all searches of pharmacophore conformations. For the representation of both the molecular electrostatic potentials and the hydrophobic potential the MOLCAD module of SYBYL was used. The molecular graphics program HAMOG (for personal computers) [29] was used for the preparation of the black-and-white figures.

Results and Discussion

Determination of the pharmacophore conformations

The μ-opioid receptor-binding conformations

The μ-opioid receptor-binding pharmacophore conformations of D-Orn^2-containing cyclic β-casomorphins have been determined previously [9–12]. Here we will describe the search for the μ-pharmacophore conformations of the remaining D-A_2bu^2 and D-Lys^2 analogues. The results of the conformational searches are summarized in Table 2.

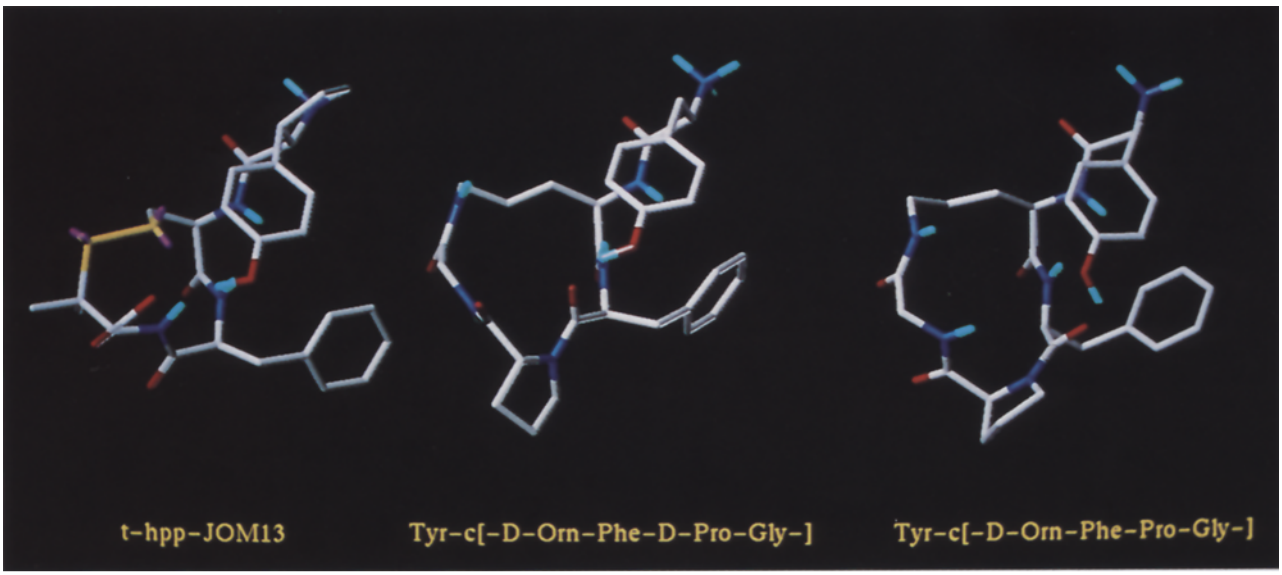

Fig. 2. The pharmacophore conformations of t-Hpp-JOM-13, H-Tyr-c[-D-Orn-Phe-D-Pro-Gly-] and H-Tyr-c[-D-Orn-Phe-Pro-Gly-].

From the molecular dynamics simulations of the Lys^2 β-casomorphin analogues we obtained on average 500 conformations for each of the *cis*- and *trans*-Pro conformations with low energy (< 7 kcal/mol), which we subsequently considered in the pharmacophore searches.

We have shown previously that the protonated nitrogen atom of opioids may interact with the anionic μ-receptor subsite in two different orientations [22]. Therefore, spatial comparisons of all low-energy conformations of D-Orn^2-containing cyclic β-casomorphins with PEO and fentanyl have been performed in a recently published paper [10]. The obtained pharmacophore conformations of the β-casomorphins correlate only with the position of the nitrogen atom of PEO and not with fentanyl. Consequently, we performed spatial comparisons of all obtained low-energy conformations (see Table 2) with PEO only. For this purpose we superimposed the centroids of both phenyl rings (Tyr^1 and Phe^3 aromatic rings) and the corresponding rings in PEO, the protonated nitrogen atoms, and the phenolic oxygen atoms as a fourth coordinate. For all D-Xaa^2 cyclic β-casomorphin derivatives several conformations with rms values lower than 0.5 Å were found. Further superpositions with more atom pairs seemed to be not worthwhile, due to several opportunities of functional groups (amide groups) of the peptides to be important for receptor binding. All conformations with low rms values were inspected manually in comparison to pharmacophore structure elements as defined in Refs. 10

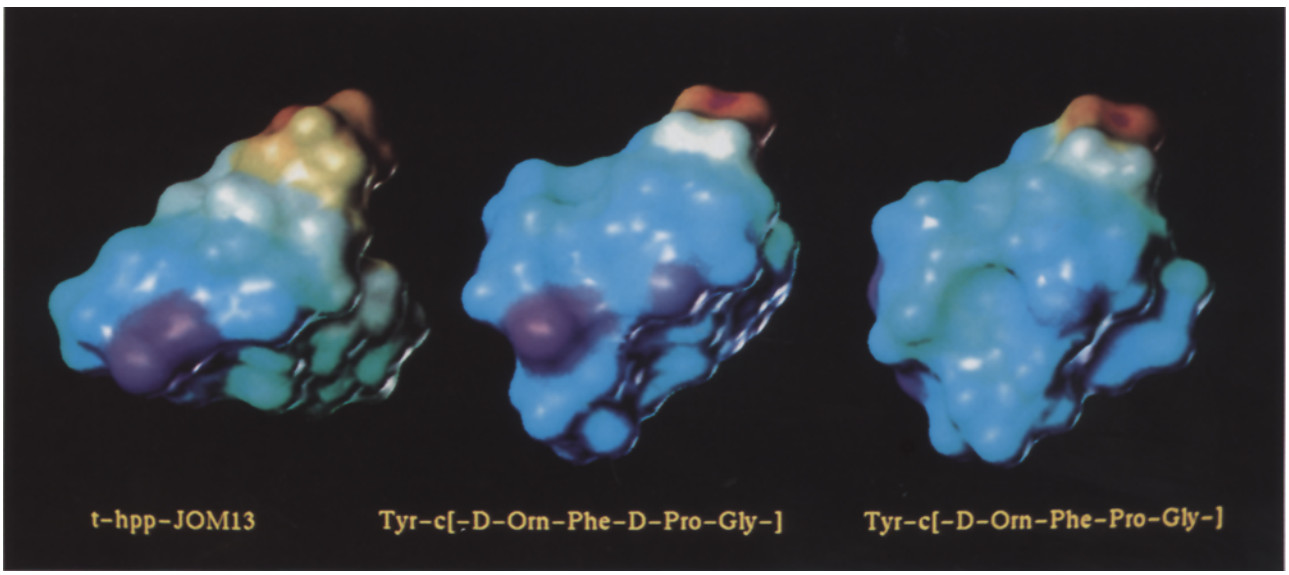

Fig. 3. Comparison of the molecular electrostatic potentials of the compounds shown in Fig. 2 (identical orientation). Red and blue indicate more positive and more negative potentials, respectively.

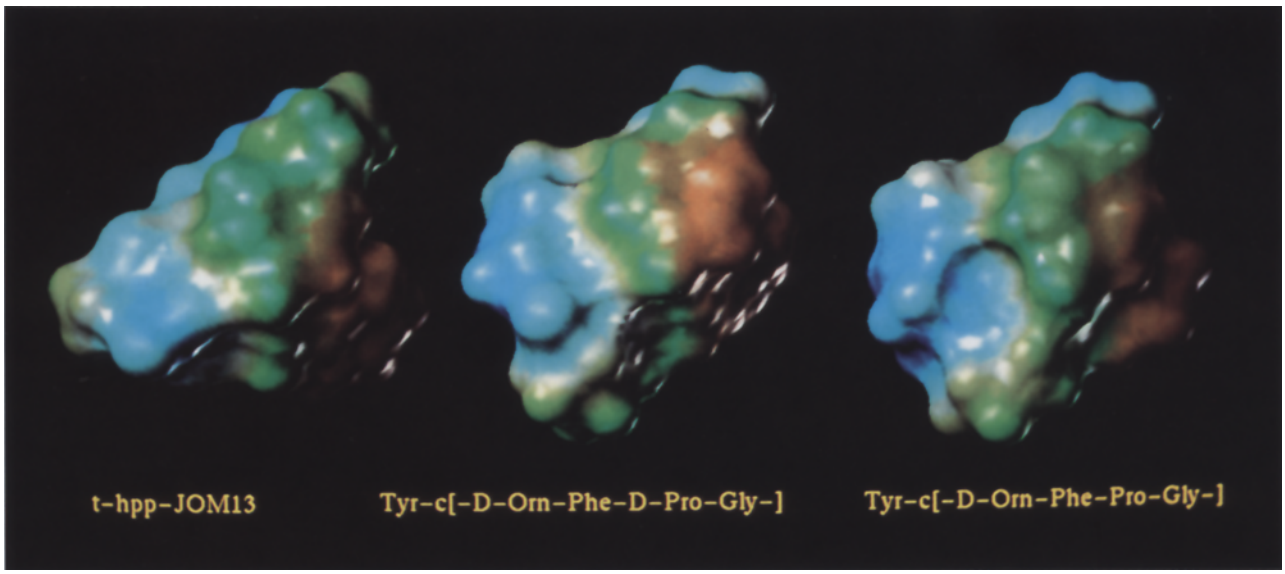

Fig. 4. Comparison of the lipophilic potentials of the compounds shown in Fig. 2 (identical orientation). Light blue represents weak lipophilicity, yellow-brown indicates a more lipophilic potential.

and 22, based on fentanyls and PEO. The proposed pharmacophoric conformation of the fentanyls was found to be very similar to results obtained by Cometta-Morini and Loew, who used the semiempirical quantum-chemical method AM1 and molecular dynamics simulations but did not perform a complete conformational search for all flexible bonds [30]. In this way we were able to pick out those low-energy conformations that very likely represent the μ-opioid receptor-binding conformations. In the case of the D-Orn^2 cyclic β-casomorphins we found a high similarity of the proposed pharmacophore conformations to the solution conformations detected by NMR spectroscopy [9–12]. The dihedral angles of these conformations of all investigated cyclic β-casomorphins are listed in Table 3.

The proposed pharmacophore conformation for H-Tyr-c[D-A_2bu-Phe-D-Pro-Gly-] in comparison to PEO and *cis*-(3*R*,4*S*,2'*S*)-ohmefentanyl is represented in Fig. 1. The areas marked A to F indicate the positions that are assumed to be essential for the interaction with the μ-opioid receptor [10,22]. Area A describes an anionic binding site, such as an aspartate or glutamate side chain, which is able to participate in an electrostatic interaction with the protonated nitrogen of the ligand in either one of the two possible orientations (compare Figs. 1a and b). The second position is area B, which interacts with the phenolic

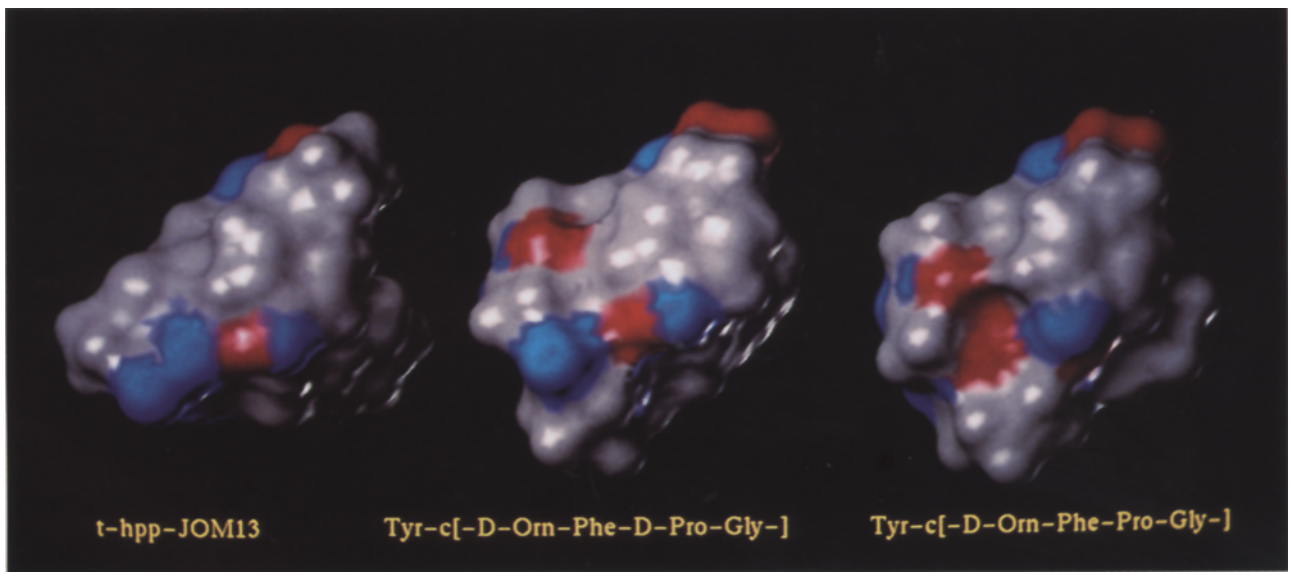

Fig. 5. Comparison of the hydrogen bonding potentials of the compounds shown in Fig. 2 (identical orientation). Red indicates proton donors, blue proton acceptor areas.

TABLE 2
NUMBER OF LOW-ENERGY CONFORMATIONS FOR CYCLIC A_2bu-CONTAINING β-CASOMORPHIN DERIVATIVES

Compound	1[a]	2[b]	3[c]
Tyr-c[D-A_2bu-Phe-*cis*-D-Pro-Gly]	65	20	43
Tyr-c[D-A_2bu-Phe-*trans*-D-Pro-Gly]	143	79	568
Tyr-c[D-A_2bu-Phe-*cis*-Pro-Gly]	124	86	1112
Tyr-c[D-A_2bu-Phe-*trans*-Pro-Gly]	228	72	866
Tyr-c[A_2bu-Phe-*cis*-D-Pro-Gly]	84	20	924
Tyr-c[A_2bu-Phe-*trans*-D-Pro-Gly]	213	108	795
Tyr-c[A_2bu-Phe-*cis*-Pro-Gly]	134	101	1027
Tyr-c[A_2bu-Phe-*trans*-Pro-Gly]	250	83	424

[a] Total number of obtained ring conformations.
[b] Number of all used low-energy ring conformations.
[c] Number of low-energy conformations of the complete molecules (up to 7 kcal/mol).

hydroxyl group of various opiates. Some opiates, including the highly active fentanyls, do not have a phenolic hydroxyl group and, therefore, do not interact with area B. Area C represents a hydrophobic receptor subsite consisting of a hydrophobic amino acid side chain such as phenylalanine, valine or proline, or groups carrying a positively charged side chain; this subsite is able to interact with an aromatic ring of the ligand in either one of several possible orientations. Area D represents another hydrophobic receptor subsite, proposed to interact with the second aromatic ring present in many ligands that show increased opioid activity. This position has been found by comparison of low-energy conformations of several opiates such as PEO, phenazocine, fentanyls, etonitazene and others [22]. Area E is a receptor subsite containing a hydrogen donor, which is able to form a hydrogen bond with the bridging oxygen atom in PEO and related opiates or with the carbonyl moiety of the fentanyls, several prodines, methadone and others. This is not in contradiction to the findings that some compounds derived from morphine but with removed oxygen bridge, e.g. levorphanol and phenazocine, show higher activity than morphine. On the one hand, these compounds are more hydrophobic, thus favoring membrane passage and reducing metabolism. On the other hand, these compounds can interact with the μ-opioid receptor in an orientation (with regard to the protonated nitrogen) as shown for *cis*-(3*R*,4*S*,2'*S*)-ohmefentanyl (Fig. 1b), which is probably favorable for higher efficacy. Area F may represent an additional hydrophilic proton-accepting binding site. For example, this area would interact with the hydroxyl group of PEO or of the ohmefentanyls. This explains the higher receptor affinity of the most active ohmefentanyl compared to fentanyl, which lacks a hydroxyl moiety. We do not exclude an additional hydrophilic binding site, which might be represented by the carboxymethyl moiety of carfentanil.

Penkler et al. [23] performed a conformational analysis of μ-selective [D-Ala^2, MePhe^4]-enkephalins using ^{1}H NMR spectroscopy and theoretical calculations. We have shown that the μ-pharmacophoric conformations suggested by these authors agree very well with those already proposed for the D-Orn^2 β-casomorphins [10,22]. Additionally, there is a high similarity to proposals for the pharmacophoric conformation of the cyclic enkephalin analog Tyr-c[-D-A_2bu-Gly-Phe-Leu-] [24]. This is also expressed by the dihedral angles $\Phi^2 = 89°$, $\Psi^2 = -36°$, which are similar to those suggested for the μ-active cyclic β-casomorphin analogues (see Table 3).

The marked areas in Fig. 1c demonstrate that all binding sites can be occupied by the cyclic β-casomorphin derivative. It seems to be important that binding site E can interact with the carbonyl oxygen atom of the Gly^4 residue. All proposed pharmacophore conformations of the active cyclic β-casomorphins adopt dihedral angles of $\Phi^2 \approx 65°$ and $\Psi^2 \approx -80°$. Such a conformation is also favorable for linear D-Pro^2 β-casomorphins. However, it has been shown that these compounds are inactive [31,32]. In our opinion the inactivity can be explained by the fact that area E is occupied by the hydrophobic pyrrolidine ring of the D-Pro^2 residue in this case. This probably causes strong repulsive interactions with the hydrophilic binding site of the receptor and, thus, prevents binding. It has been demonstrated that in the case of linear L-Pro^2 β-casomorphins or morphiceptins, a *cis*-Pro peptide bond represents likely an essential pharmacophoric pattern [10]. This has recently been supported by investigations of N^2-methylated deltorphin analogues [33], where the *cis*-Pro^2 peptide bond leads to an opposite orientation of the pyrrolidine ring, which is no longer in the hydrophilic binding area E (for more details see Ref. 10).

The selected conformations of the D-A_2bu^2 and D-Lys^2 cyclic β-casomorphin analogues are very similar to those of the corresponding D-Orn^2 derivatives. Therefore, the experimental results obtained for the D-Orn^2 analogues support the hypothesis that the D-A_2bu^2 and D-Lys^2 cyclic β-casomorphin derivatives are the μ-pharmacophores. Interestingly, the relation of the *trans*- to *cis*-Pro^4 isomers is the same as found for the ornithine-containing casomorphin analogues. In the case of L-Pro^4 derivatives, in all compounds a *cis*-Pro^4 peptide bond is energetically favored, whereas it is *trans* for the D-Pro^4 analogues. In spite of the different side-chain length of the residues in position 2, the relevant distances between pharmacophorically important groups are nearly identical in all μ-pharmacophore conformations (see Table 3, distances d_1 and d_2).

The δ-opioid receptor-binding conformations

To establish the δ-pharmacophoric conformations among the manifold of conformations of the cyclic β-casomorphins, we compared all low-energy conformations with regard to spatial similarities with the proposed δ-pharmacophore conformations of t-Hpp-JOM-13 [18,19].

We took into account the possible conformations of χ^1 of phenylalanine in t-Hpp-JOM-13 to be 60° or −60° both of which were considered to represent the δ-pharmacophoric conformation [18,19]. For this purpose, first, we used three pairs of coordinates for the comparison. These are the centroids of the Phe^3 aromatic rings, the phenolic oxygen atoms and the position of the N-terminal protonated nitrogen atoms. The results of these superpositions are listed in Table 4 (fit1). These results did not discriminate between the active and inactive compounds.

The interaction of pharmacophore groups with the receptor is one important requirement for high analgesic potency of compounds. However, the overall shape of the molecules should also match the binding site without spatial hindrance. To compare the shape of the target t-Hpp-JOM-13 with the β-casomorphins, we added the atoms of the peptide bonds of the first three amino acid residues ($C^{1'}$, O^1, N^2, $C^{2'}$, O^2, N^3, $C^{3'}$, O^3) to those used in fit1 (see Table 4) and performed another run of superpositions (Table 4, fit2). Now, a more obvious difference in the rms values between the active and inactive compounds can be seen. These differences have been obtained using exactly the same procedure for the active and inactive compounds in the superpositions with t-Hpp-JOM-13. Therefore, these results indicate indirectly that the applied procedure and the used target compound may reflect the main properties of the δ-pharmacophore conformations. In this way, we could select the likely δ-pharmacophore conformations of all considered β-casomorphin derivatives. The dihedral angles of the predicted δ-pharmacophore conformations of all regarded active cyclic β-casomorphin analogues are listed in Table 3. For comparison, we include the torsional angles proposed by Mosberg et al. [18,19] for t-Hpp-JOM-13. For a conformation of t-Hpp-JOM-13 with χ^1 of Phe$^3 \approx 60°$, such a clear result could not be obtained. Therefore, our results support the assumption that the conformation of t-Hpp-JOM-13 with χ^1 of Phe$^3 = -59°$ more likely represents the

TABLE 3

TORSION ANGLES AND RELATIVE ENERGIES OF POSSIBLE μ- AND δ-PHARMACOPHORE CONFORMATIONS OF CYCLIC β-CASOMORPHIN ANALOGUES H-Tyr-c[-X-Phe-Y-Gly-]

Residue	X = D-A_2bu, Y = Pro		X = D-A_2bu, Y = D-Pro		X = D-Orn, Y = Pro		X = D-Orn, Y = D-Pro		X = D-Lys, Y = Pro		X = D-Lys, Y = D-Pro		t-Hpp-JOM-13[a]
	δ	μ	δ	μ	δ	μ	δ	μ	δ	μ	δ	μ	δ
Tyr^1													
Ψ	157	154	162	151	156	148	159	153	164	153	158	148	155
χ^1	176	175	−176	178	174	176	−179	−178	−172	175	179	179	−158
χ^2	76	85	54	82	76	87	77	79	69	85	75	91	61
X^2													
Φ	161	70	165	63	164	68	166	65	164	70	165	73	158
ψ	56	−87	−55	−80	57	−87	59	−87	51	−88	64	−65	39
χ^1	172	176	−40	−39	171	169	172	−75	−178	171	−168	−77	−57
χ^2	74	64	−55	−46	167	54	176	61	−166	68	−176	146	−148
χ^3		78		106	−72	56	−68	−76	−169	69	−176	−83	94
χ^4						−162		153	59	−148	64	−61	
χ^5										−4		109	
Phe^3													
Φ	−165	−47	−52	−63	−169	−66	−140	−71	−171	−58	−141	−79	−85
Ψ	136	134	144	138	138	136	149	138	82	142	158	129	−40
ω	1	3	179	−176	−3	−2	178	−179	−4	−1	179	172	
χ^1	52	180	64	177	51	−179	−51	175	50	178	−55	177	−65
χ^2	88	95	85	96	91	94	108	95	97	97	110	90	88
Y^4													
Φ	−70	−76	78	56	−77	−72	48	58	−79	−82	83	81	141
Ψ	−12	−11	−71	40	−17	136	36	54	−170	158	−64	18	−70/53[b]
Gly^5													
Φ	−134	−145	−127	116	−84	97	159	134	−137	70	−164	144	
Ψ	88	72	−51	71	−90	−126	−121	1.4	94	−74	80	107	
ΔE	0.32	5.2	5.50	4.1	3.68	3.5	3.38	4.5	1.21	2.1	0.00	4.8	
d_1	6.62	8.0	6.73	6.9	6.74	7.2	6.43	7.2	5.83	7.8	6.45	6.9	7.41
d_2	4.59	11.3	5.11	10.9	5.11	11.1	5.56	11.0	4.93	11.3	5.57	10.8	6.17

Distances (in Å): d_1 = the distance between the N-terminal nitrogen atom and the centroid of the aromatic ring in position 3; d_2 = the distance between the centroids of the aromatic rings. Energies are given in kcal/mol, dihedral angles in degrees.

[a] Taken from Ref. 18.

[b] Values are related to χ^1 and χ^2.

δ-pharmacophore conformation. The comparison of the best-matching conformers of the cyclic β-casomorphins with t-Hpp-JOM-13 obviously shows a very high similarity (see Fig. 2). This is also expressed by the formation of a hydrogen bond between the phenolic hydroxyl group and the carbonyl oxygen atom of Phe[3]. However, the orientation of this hydrogen bond is equivalent to the one in t-Hpp-JOM-13 only for the D-Pro[4] β-casomorphin analogues, whereas it is inverted in the L-Pro[4] derivatives (see for example Fig. 2).

Spatial similarities of biologically active compounds are the primary requirement for similar activity, but not a sufficient one. The ability to form hydrogen bonds with the receptor in defined receptor positions as well as a similar distribution of the electrostatic and hydrophobic potentials are additional important properties for similar biological behavior of different compounds. Therefore, we compared the hydrogen bonding, electrostatic and lipophilic potentials of the δ-pharmacophore conformations of the compounds considered in this study with each other. These are presented in Figs. 3 to 5. The high similarity of the potentials to each other and to t-Hpp-JOM-13 strongly supports the correctness of our predictions of the δ-pharmacophore conformations of the cyclic β-casomorphins. In particular, we find a negative potential in the vicinity of the C-terminal carboxyl group of t-Hpp-

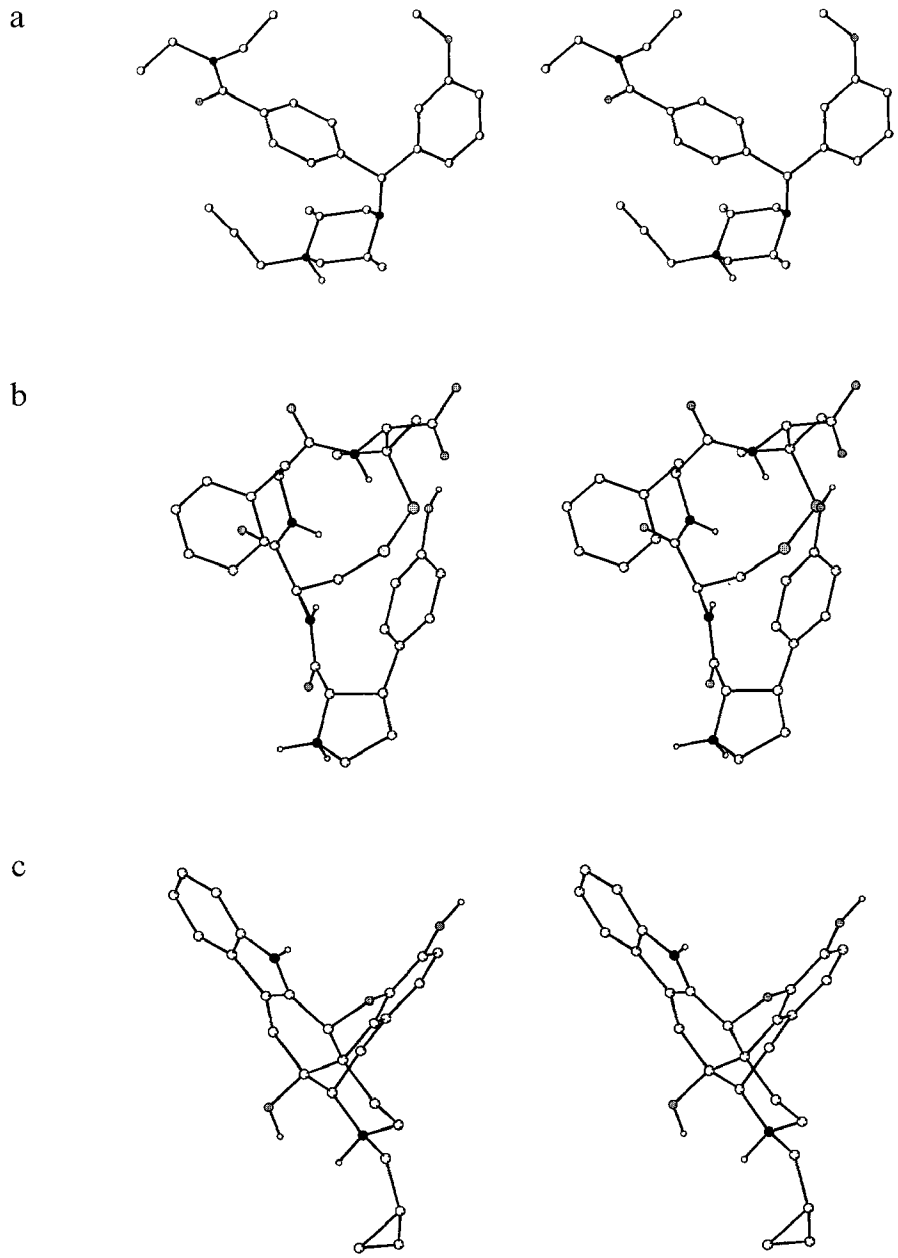

Fig. 6. Stereo representation of the best matching conformations of (a) SNC 80; and the proposed δ-pharmacophores of (b) t-Hpp-JOM-13; and (c) naltrindole.

TABLE 4
RESULTS OF SUPERPOSITIONS OF CYCLIC β-CASOMORPHIN DERIVATIVES (Tyr-c[-Xaa-Phe-Yaa-Gly-]) WITH t-Hpp-JOM-13

No.	Substitution		Fit1[a]		Fit2[b]	
	Xaa	Yaa	Rms (Å)	ΔE (kcal/mol)	Rms (Å)	ΔE (kcal/mol)
1	D-A_2bu	Pro	0.254	5.54	0.722	0.32
2	D-A_2bu	D-Pro	0.167	5.17	0.770	5.50
3	D-Orn	Pro	0.112	1.96	0.540	3.68
4	D-Orn	D-Pro	0.278	3.35	0.504	3.38
5	D-Lys	Pro	0.521	11.34	0.432	1.21
6	D-Lys	D-Pro	0.275	7.20	0.535	0.00
7	A_2bu	Pro	0.116	2.00	1.434	7.04
8	A_2bu	D-Pro	0.226	5.92	1.636	5.83
9	Orn	Pro	0.133	5.36	1.411	5.24
10	Orn	D-Pro	0.112	3.94	1.319	6.78
11	Lys	Pro	0.308	13.53	0.972	10.34
12	Lys	D-Pro	0.110	4.69	0.951	8.97

[a] Fit1 is a superposition of three atom pairs (phenolic **OH**, centroid of the Phe^3 aromatic rings, **N** of the N-termini).
[b] Fit2 is a superposition of 11 atom pairs (in addition to those of fit1, all atoms of the peptide bonds of the first three amino acid residues).

JOM-13, which agrees with a negative potential of H-Tyr-c[-D-Orn-Phe-D-Pro-Gly-] formed by the carbonyl oxygen atoms of residues 3 and 4. This β-casomorphin analogue exhibits the highest δ-opioid binding affinity. The weaker δ-opioid receptor-binding β-casomorphin derivatives do not show such a high similarity in this area. This allows the more general conclusion that this area plays an important role in the interaction with a hydrophilic proton donor or cationic binding site of the δ-opioid receptor. This hypothesis is supported more indirectly by the findings of Schiller et al. [34], who investigated dermorphin analogues with positively charged side-chain residues, such as A_2bu, Orn and Lys, in position 4. All these compounds show high selectivity for the μ-opioid receptor and only weak affinity to the δ-opioid receptor. These results are in agreement with suggestions of Schwyzer [35], who demonstrated that positive charges within the message sequence of opioids increase μ-selectivity, whereas the introduction of negatively charged groups leads to preferred binding to the δ-opioid receptor. Melchiorri et al. [35] investigated a number of deltorphin derivatives. Most of these compounds, not possessing a negatively charged amino acid side chain in position 4 or 5 of the dermorphin sequence, show μ-opioid receptor selectivity. Introduction of Asp^4 or Glu^4, however, leads mostly to highly selective δ-opioid receptor ligands. It can be as-

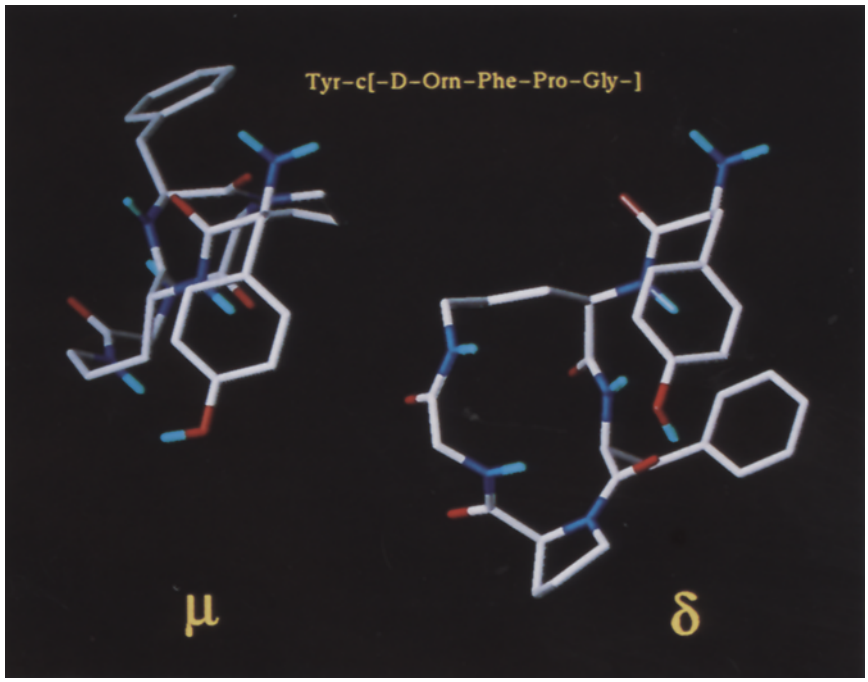

Fig. 7. Comparison of the μ- and δ-pharmacophore conformations of H-Tyr-c[-D-Orn-Phe-Pro-Gly-] with superposition of the tyrosine residues.

sumed that these compounds will agree with the three-dimensional structure and surface potentials of the compounds investigated here. Exceptions from this rule are compounds like Asp^4-Tyr^5-deltorphin analogues or derivatives with a positively charged amino acid residue in position 5. In these cases, we assume a strong electrostatic interaction between the side chains of Asp^4 and the residues in position 5, which prevents the adoption of the suggested δ-pharmacophore conformation. The most potent and most selective δ-agonist deltorphins, D-Ala^2-deltorphin I and D-Ala^2-deltorphin II, contain an aspartate or glutamate residue in their sequence, too. Similar results were obtained by Lazarus et al. [37], who showed that the location of the negatively charged groups relative to hydrophobic residues in the address domain of deltorphins is of high importance for both δ-affinity and selectivity. Since this property was never considered in the superpositions of the investigated compounds, the agreement of the negative potential of the cyclic β-casomorphin derivative with the highest δ-affinity among them to the one in t-Hpp-JOM-13 is particularly remarkable. Thus, this result might be in strong support of the suggested δ-pharmacophore conformations of the β-casomorphins. Stereospecific design of new cyclic β-casomorphin derivatives based on these results will show whether or not the conclusions are correct.

It has been suggested that peptide opioids bind to different binding sites of the δ-receptor compared to nonpeptide opioids as well as antagonists [21,38,39]. Therefore, we try to answer the question whether this is also expressed by different spatial arrangements of the pharmacophore groups and surface potentials. A highly active nonpeptide δ-opioid receptor agonist (SNC 80) has been synthesized and tested by Calderon et al. [20]. Portoghese et al. [21] synthesized relatively rigid naltrexone-derived δ-opioid antagonists. We included these compounds in our theoretical investigation to unravel similarities between the pharmacophore conformations of the cyclic β-casomorphin analogues and t-Hpp-JOM-13. Complete conformational analysis of the peptides has been performed as described before by varying each flexible torsion angle in 30° increments. All obtained low-energy conformations were compared with the peptide pharmacophore, taking into account the protonated nitrogen atoms and the centroids of the aromatic phenyl rings. In each case we could find a similarity with the δ-pharmacophoric conformations of the peptides. This is shown in Fig. 6 for SNC 80, NTI and t-Hpp-JOM-13. It is possible to superimpose the aforementioned groups, but obviously a negatively charged group in the suggested area could never be detected. Furthermore, the planes of the aromatic rings are more-or-less twisted with regard to those in t-Hpp-JOM-13. Interestingly, the distances between the centroids of the aromatics and to the protonated nitrogen atom are very similar to those found for the peptides. An exception is SNC 80, where the distance between the centroids is about 2 Å shorter than in the peptides. The possibility cannot be excluded that in this case one of the ethylene groups linked to the amide bond will interact with a hydrophobic binding site rather than the phenyl ring. However, a better match with the backbone atoms of δ-pharmacophoric conformations of opioid peptides could not be detected. This either supports the above discussed suggestions by other authors [21,38,39] for different binding domains of peptides, nonpeptides and antagonists or it offers new chances to design higher δ-selective and active nonpeptide agonists or antagonists. The latter seems to be possible for instance by introduction of negatively charged groups. These groups should adopt exactly the same position as derived from the comparison of the compounds shown in Fig. 6.

The predicted δ-pharmacophore opioid binding conformations of the cyclic β-casomorphins should be compared now with the already proposed μ-pharmacophore one. By comparing the dihedral angles listed in Table 3, similarities as well as differences can be found. Thus, the Tyr^1 residues of both the μ- and δ-pharmacophores adopt nearly the same conformation, characterized by the dihedral angles χ^1, χ^2 and Ψ^1 which are about 170°, 80° and 140°, respectively. Moreover, the relations of the *cis/trans*-Pro conformation are exactly identical. The high similarity in the Tyr^1 part of the address sequence of the opioid peptides may be the main reason for interaction of peptides with both receptors. The remaining torsional angles of the backbone, however, differ essentially between the μ- and δ-pharmacophores. In particular, alterations in Φ^2 and Ψ^2 cause a change in the direction of the carbonyl group of residue 2. We have demonstrated that the backbone conformation (γ-turn formation) of the second residue of μ-selective compounds seems to play an important role in receptor recognition [10]. The δ-pharmacophore conformations in all cyclic β-casomorphins and in t-Hpp-JOM-13 exhibit also a high similarity at residue 2. However, the dihedral angles Φ^2 and Ψ^2 differ essentially compared to the related dihedral angles of the μ-conformations. Therefore, it may be suggested that, besides the orientation and distances of the aromatic groups (distances d_1 and d_2), this is an additional essential feature (very likely for the formation of hydrogen bonds with the receptor) for both the μ- and δ-opioid receptor-binding pharmacophores.

Assuming that both the μ- and δ-pharmacophoric conformations bind with the tyrosine residue in a similar orientation at the same transmembrane domain X of their receptors, then, for instance, the side chain of Phe^3 as a second binding site should dock with different domains. For reasons of clarity, we represent in Fig. 7 the μ- and δ-pharmacophoric conformations of H-Tyr-c[-D-Orn-Phe-Pro-Gly-], keeping the orientation of the tyrosine residues fixed.

Conclusions

We have shown that cyclic β-casomorphin derivatives with a D-amino acid residue in position 2 are able to adopt both μ- and δ-opioid receptor-binding conformations. These conformations are characterized by distinct arrangements of two aromatic rings and a protonated N-terminal nitrogen atom. However, it turned out that the widely accepted importance of these pharmacophoric groups is not the only essential requirement for opioid activity and selectivity. For the μ-receptor-binding conformations of the β-casomorphins, we were able to demonstrate that hydrophilic binding sites in the vicinity of the Pro^4 and Gly^5 positions of the cyclic β-casomorphins, characterized by spatial comparisons with PEO and fentanyl analogues, are also important for receptor affinity and selectivity. Similar results were obtained for the δ-pharmacophoric conformations with regard to the importance of the spatial arrangements of the overall backbone structure of the active β-casomorphin analogues. This could be demonstrated by the two steps of superpositions with t-Hpp-JOM-13. When we took into account several atoms of the backbones, a clear differentiation between active and inactive β-casomorphin analogues could be made. In particular, the comparison of surface potentials of the molecular electrostatic potentials led to the definition of an area where negative potential should be produced by ligands to bind selectively to the δ-opioid receptor.

Moreover, the differences in the dihedral angles Φ^2 and Ψ^2 for the μ- and δ-pharmacophoric conformations ($\mu \approx 70°,-80°$; $\delta \approx 165°,55°$) cause a completely different spatial arrangement of the cyclized peptide rings when all compounds are superimposed with regard to maximal spatial overlap of the tyrosine residue (see Fig. 7). In our opinion, this difference is as important as larger distances between the centroids of the aromatics Tyr^1 and Phe^3 for the μ-selective conformations (10 to 11 Å) in comparison with the δ-conformations (5 to 7 Å). Assuming that both the μ- and δ-pharmacophoric conformations bind with the tyrosine residue in a similar orientation at the same transmembrane domain X of their receptors, then e.g. the side chain of Phe^3 at a second binding site should dock with different domains. The synthesis of compounds that include peptide mimetics with fixed dihedral angles Φ^2 and Ψ^2 may lead to compounds with improved selectivity and can serve to prove the model.

The analysis of very likely important binding sites of the suggested μ- and δ-pharmacophoric conformations to their receptors additionally offers a manifold of new ideas to synthesize compounds with improved selectivity and affinity to the opioid receptors. On the basis of the results and suggestions described here, stereoselective modifications of relatively rigid compounds such as the cyclic β-casomorphins or compounds derived from other classes of opioid peptides as well as nonpeptide agonists or antagonists will gain more insights into ligand–opioid receptor binding requirements.

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