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Prerequisite for His[4] in deltorphin A for high δ opioid receptor selectivity

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Summary. Analysis of deltorphin A position 4 analogues included: backbone constrained N$^{\alpha}$MeHis, spinacine (Spi), N$^{\alpha}$MePhe and the tetrahydroisoquinoline-3-carboxylic acid (Tic); spatially confined side-chain (Phg); and imidazole alkylation of L- and D-His[4] enantiomers. High δ selectivity was lost with the following replacements: N$^{\alpha}$MeHis[4], N$^{\alpha}$MePhe[4] and Phg[4] reduced δ binding and the constrained residues also increased μ binding; ring closure between the side-chain and amino group to yield Spi[4] or Tic[4] increased μ affinity. Imidazole methylation of His[4] marginally affected opioid binding and doubled δ selectivity; alkylated D-His[4]-derivatives generally maintained δ selectivity in spite of decreased δ affinities. Thus, His[4] imidazole preserves δ selectivity by facilitating high δ binding and by repulsion at the μ receptor. Several low energy conformers of deltorphin A indicated that the His[4] imidazole preferred a spatial orientation parallel to the phenolic side-chain of Tyr[1] suggestive that this conformation might contribute to high δ affinity and selectivity.

Keywords: Amino Acids – Deltorphins – Peptide synthesis – Opioid receptors – Molecular dynamics simulations

Introduction

Amphibian skins are well known for their abundance and diverse array of bioamines, toxins and alkaloids (Erspamer, 1984; Daly et al., 1987), in addition to biologically active proteins and peptides which encompass the entire spectrum of neuropeptides and gastrointestinal peptides associated with mammalian tissues (Erspamer, 1984; Lazarus and Attila, 1993). The biological properties of

these peptides reflect an even greater diversity than the numerical quantity of the peptides themselves (Lazarus and Attila, 1993). Of this treasure trove of peptidic substances, however, one of the most interesting and unique classes of amphibian skin peptides, the opioid heptapeptides, entails the acquisition of a rare D-amino acid enantiomer at the second position (Montecucchi et al., 1981a, b; Erspamer, 1992). The presence of this unusual configuration enables these peptides to exhibit exceptionally potent opioid properties (Erspamer, 1992). The opioid peptides are subdivided into two groups based upon their selectivity for either μ or δ receptor sites, since none interact to any appreciable degree with κ receptors (Erspamer, 1992): dermorphins are μ selective compounds, while deltorphins exhibit high selectivity for δ receptors. The most interesting structural feature of these opioid peptides lies in the common motif of their N-terminal tripeptide sequence: namely, H-Tyr-D-Xaa-Phe (D-Xaa^2 is either D-Ala or D-Met), which is characterized by a type II′ β-turn in solution (Pattabiraman et al., 1986; Tancredi et al., 1991; Amodeo et al., 1992; Ohno et al., 1993).

The first recognized δ opioid peptide from amphibian skin, deltorphin (H-Tyr-D-Met-Phe-His-Met-Asp-NH_2), was initially detected in a cDNA clone for dermorphin (Richter et al., 1987) and termed dermorphin gene-related peptide (Lazarus et al., 1989b), dermenkephalin (Mor et al., 1989) and deltorphin (Kreil et al., 1989), and subsequently renamed deltorphin A (Lazarus et al., 1991). Interestingly, the cDNA sequence of this peptide and the multiple dermorphin transcripts coded only for L-amino acids (Richter et al., 1987). Ensuing peptide synthesis and actual isolation (Mor et al., 1989; Kreil et al., 1989), coupled with receptor binding characteristics and bioactivity profiles, revealed that the active conformer required a D-enantiomer at position 2 since L-Met^2 was essentially inactive (roughly three orders of magnitude less effective) (Lazarus et al., 1989b; Mor et al., 1989; Kreil et al., 1989). Two additional δ opioid specific peptide members of this group emerged shortly afterward that contained D-Ala^2 (Erspamer et al., 1989; Richter et al., 1990) and whose C-terminal tetrapeptide sequence differed from that of deltorphin A, the [D-Ala^2]deltorphins I and II (Erspamer et al., 1989) or the comparable deltorphins C and B, respectively (Lazarus et al., 1991).

Structure-activity studies on the deltorphins indicated that the spatial orientation and conformational integrity of the N-terminal tripeptide sequence is crucial for the expression of opioid activity (Schiller et al., 1990; Salvadori et al., 1991; Lazarus et al., 1991, 1992a, 1993). The systematic application of D-amino acid replacements in deltorphin A succinctly revealed the further importance of the spatial configuration of each residue in the peptide (Lazarus et al., 1992). Deletion of His^4 (Lazarus et al., 1992a) or its replacement by residues with charged, aromatic (Salvadori et al., 1991; Sagan et al., 1992; Lazarus et al., 1993) or hydrophilic side-chains (Misicka et al., 1991) suggested that His^4 either plays a direct role in the overall conformation of the peptide or serves merely as a linker between the N- and C-terminal regions, the "messenger" and "address" domains according the model on opioid peptides and naltrindole developed by Portoghese (1989, 1993). Until the differences and similarities in the amino acid sequence and the tertiary structure of the specific δ and μ binding sites are

known, even though the μ receptor contains 61%–64% sequence similarity (Chen et al., 1993; Fukuda et al., 1993) to that of the δ receptor (Evans et al., 1992; Kieffer et al., 1992; Fukuda et al., 1993; Yasuda et al., 1993), our studies provide an essential approach to experimentally define specific peptide residues and potential receptor binding sites.

This communication, therefore, focuses on an attempt to refine our perception of the specific function of the imidazole side-chain and the contribution of the amino backbone of His[4] on binding to δ and μ opioid receptor sites. This strategy was pursued employing the replacement of His[4] in deltorphin A analogues uniquely designed to impose a constraint on peptide conformation and on the spatial dimensions of the side-chain at position 4. The observed receptor affinities were then considered in relation to a low energy conformer of deltorphin A determined by the principles of molecular dynamics simulations for solvated deltorphins established by Bryant et al. (1993) and explored by others (Nikiforovich et al., 1991, 1993; Ohno et al., 1993).

Materials and methods

Enkephalin-derived peptides, cyclic [D-Pen[2,5]] (DPDPE) and [D-Ala[2],N$^{\alpha}$MePhe[4],Gly-ol[5]] (DAGO) were obtained from Bachem (Torrance, CA); the radioisotopic equivalents, [^{3}H]DPDPE (60.0 Ci/mmol) was from Amersham (Arlington Heights, IL) and [^{3}H]DAGO (37.1 Ci/mmol) from NEN-DuPont (Billrica, MA). The following items were purchased from Sigma Chemical Co. (St. Louis, MO): bacitracin, bestatin, bovine serum albumin (RIA grade), HEPES (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid), phenylmethylsulfonyl fluoride, soybean trypsin inhibitor and ultrapure sucrose. Glass microfibre (GF/C) filters were a product of Whatman International, Ltd. (Maidstone, United Kingdom). Amino acids and protected amino acids containing the protecting groups Fmoc [N^{α}-(9-fluorenylmethyl) oxycarbonyl], Boc (N^{α}-*tert*-butoxycarbonyl), O*t*Bu (*tert*-butyl ester), OBzl (benzyl ester) and OMe (methyl ester) were products of Bachem (Switzerland) and Senn Chemicals AG (Germany) and the solid support Fmoc-PAL-PEG-PS resin was from Millipore (Waltham, MA). All other chemicals were of the highest purity available and obtained from several reliable sources.

Peptide synthesis

Solid-phase synthesis

Deltorphin A was prepared by solid-phase methods described in detail elsewhere (Lazarus et al., 1991, 1993; Salvadori et al., 1992). Peptides **10–12** (Table 1) were synthesized by solid phase methods using Fmoc-PAL-PEG-PS (0.18 mmol/g; 0.09 mmol) with a Milligen 9050 peptide synthesizer.

N$^{\alpha}$Fmoc-amino acid derivatives were used in the coupling reactions. The reactive side-chain of Asp was protected with *tert*-butyl ester, while Tyr was protected with *tert*-butyl ether. Amino acids were coupled in a 4-fold excess using diisopropylcarbodiimide (DIPCDI) in the presence of a 4-fold excess of hydroxybenzotriazole (HOBt) for 1 hr. Coupling was monitored by absorbance of the Fmoc derivatives. Deprotection of amino protected intermediates on the resin was accomplished with 20% (v/v) piperidine in N,N′-dimethylformamide (DMF). The step related to the acylation of N$^{\alpha}$MePhe and Tic requires double coupling and the synthesis of compound **10** was carried out in the DMF solution containing 0.4 M LiCl. Protected peptides were cleaved from the resin by treatment with 15 mL/g resin modified reagent B (88% TFA, 5% H_2O and 7% triethylsilane) for 1 hr at room temperature.

Table 1. Analytical data on His[4]-substituted deltorphin A analogues

No.	Peptide	TLC, R_f		HPLC	FAB-MS	Amino acid analysis				
		I	II	K′	MH+	Y	M	F	L	D
2.	[His(1-Me)[4]]	0.53	0.45	5.78	969	1.01	1.94	1.0	0.97	1.0
3.	[D-His(1-Me)[4]]	0.55	0.41	4.86	969	0.95	1.99	0.97	1.02	1.0
4.	[His(3-Me)[4]]	0.51	0.45	5.56	969	0.98	2.01	1.02	1.02	1.0
5.	[D-His(3-Me)[4]]	0.53	0.46	4.79	969	0.95	1.97	0.99	1.03	1.0
6.	[N[α]MeHis[4]]	0.72	0.67	6.27	969	0.97	2.01	1.02	0.99	1.0
7.	[N[α]MeHis[4],Asp(OBzl)[7]]	0.83	0.72	7.56	1059	0.95	1.97	0.98	0.99	1.0
8.	[Spi[4]]	0.49	0.28	6.71	967	0.89	2.02	0.97	1.01	1.0
9.	[Spi[4],Asp(OMe)[7]]	0.61	0.43	7.68	981	0.95	1.98	0.95	0.97	1.0
10.	[N[α]MePhe[4]]	0.78	0.66	9.75	979	0.97	2.02	0.95	1.01	1.0
11.	[Tic[4]]	0.73	0.60	9.66	977	0.89	1.99	1.02	1.01	1.0
12.	[Phg[4]]	0.85	0.74	9.85	951	0.97	2.03	1.02	0.99	1.0

Solution synthesis

Peptides **2–9** were prepared by solution synthesis involving tetra- and tripeptides (4 + 3 coupling) as detailed in Fig. 1. The N-terminal tetrapeptides Boc-Tyr-D-Met-Phe-Xaa-OH were prepared step-wise starting with His-containing analogues, where Xaa = L- or D-His(1-Me), L- or D-His(3-Me), N^{α}MeHis or Spi, using active ester condensation with N-hydroxysuccinimidyl ester (OSu). In the reaction involving N^{α}-alkylated amino acids, N^{α}MeHis and Spi, we obtained good yields of the dipeptides Boc-Phe-N^{α}MeHis-OH and Boc-Phe-Spi-OH by using a 5-fold excess of the amino component without extraneous side reactions on the imidazole nucleus. The Boc protecting group was removed by 50% TFA in CH_2Cl_2 at 0 °C.

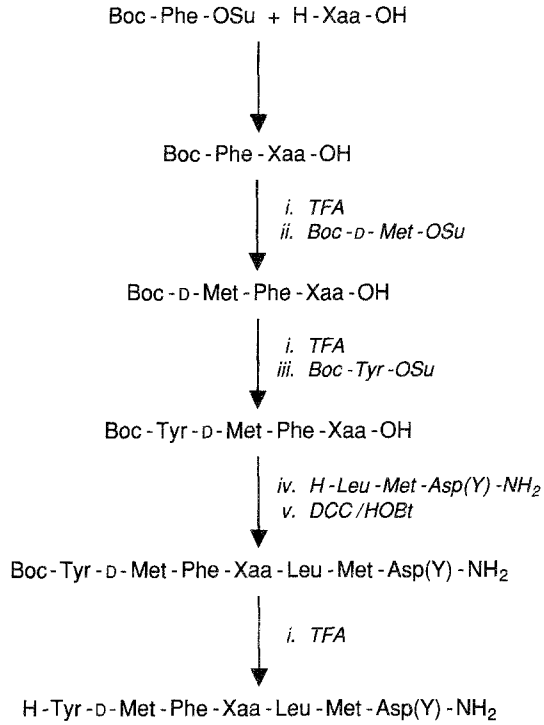

Fig. 1. Schematic representation of the synthesis of His[4]-substituted deltorphin A analogues **2–9**. *Xaa*, L- or D-His(1-Me), L- or D-His(3-Me), N^{α}MeHis and Spi; *Y*, O*t*Bu, OBzl, OMe or OH. The numbers indicate the substances used at each step in the synthesis

The synthesis of the C-terminal tripeptide intermediate H-Leu-Met-Asp(O*t*Bu)-NH_2 was described earlier (Tancredi et al., 1991). The C-terminal tripeptides of compounds **7** and **9** were prepared from H-Asp(OBzl)-OH and H-Asp(OMe)-OH followed by protection of the α-amino group with di-*tert*-butyldicarbonate (Boc) and amidation of α-carboxy function by mixed anhydride activation and reaction with ammonia to give Boc-Asp(Y)-NH_2 (Y = OBzl or OMe). Boc deprotection (50% TFA in CH_2Cl_2 at 0 °C) was followed by two steps of OSu condensation, Boc-Met-OSu and Boc-Leu-OSu, gave the protected tripeptides Boc-Leu-Met-Asp(Y)-NH_2. The final 4 + 3 condensation was obtained in good yield using dicyclohexylcarbodiimide (DCC) in the presence of HOBt.

Peptide purification

All protected peptides (0.7–1 g) were initially purified on a silica gel column (2 × 70 cm); elution was carried out using a linear gradient from 10% to 50% MeOH in $CHCl_3$.

Deprotected peptides were further purified by a combination of Sephadex gel filtration, partition chromatography and preparative HPLC. Preparative HPLC of 100 mg quantities was performed on a Delta Pak reverse-phase C18 300 Å 15 μm spherical particle column (10 × 300 mm); elution was conducted using a linear gradient from 20% to 50% acetonitrile containing 0.1% TFA over 40 min at a flow rate of 30 mL/min.

Analytical determinations

Analytical HPLC utilized a Bruker liquid chromatography LC 21-C instrument fitted with a Vydac C18 218 TP 5415 5 μm particle column (4.5 × 175 mm) and equipped with a Bruker LC 313 UV variable wavelength detector. Mobile phases A and B were composed of 10% acetonitrile in 0.1% TFA and 60% acetonitrile in 0.1% TFA, respectively. Recording and quantification were accomplished with a chromatographic data processor coupled to an Epson computer system (QX-10). Analytic determinations and capacity factors (K′) of the peptides were determined with a linear gradient of 10% B to 60% B at a flow rate of 1 mL/min in 25 min.

Amino acid analyses used the Pico-Tag work station (Waters-Millipore, Waltham, MA) with PITC as the amino acid derivatization reagent. Lyophilized samples of peptides (50–1000 pmol) were sealed in heat-treated borosilicate tubes (4 × 50 mm) and hydrolyzed in 200 μL 6 N HCl containing 1% phenol in the Pico-Tag work station for 1 hr at 150 °C. A Hypersil ODS 5 μm particle size column (4.6 × 250 mm) was employed to separate the PITC-amino acid derivatives.

TLC used precoated plates of silica gel F-254 (Merck, Darmstadt, Germany) in the following solvent systems: I, 1-butanol/acetic acid/H_2O (3:1:1, v/v/v); II, ethyl acetate/pyridine/acetic acid/H_2O (12:4:1.2:2.2, v/v/v/v); III, CH_2Cl_2/MeOH/toluene (8.5:1.0:0.5, v/v/v); and IV, $CHCl_3$/MeOH/benzene/H_2O (8:8:8:1, v/v/v/v). Ninhydrin (1%), fluorescamine and chlorine spay reagents were employed to detect the peptides.

All analogues revealed less than 1% impurities when monitored at 220 nm. Chemical characteristics were confirmed using ^{1}H-NMR spectroscopy with a 200 MHz Bruker instrument. Molecular weights of peptides were determined by FAB mass spectrometry on a VG ZAB 2 SE double focusing mass spectrometer equipped with a caesium gun operating at 2 μA (20 KV); the sample was dissolved in a 5% acetic acid and loaded onto a glycerolthioglycerol coated probe tip.

Radioreceptor assays

The affinities of the deltorphin A analogues were determined in competitive binding assays using [^{3}H]DPDPE (6.0 nM) to label the δ sites and [^{3}H]DAGO (1.28 nM) for the μ sites according to published methods (Lazarus et al., 1992, 1993; Salvadori et al., 1992). Excess unlabelled peptides (2 μM) were used to saturate the opioid binding sites in order to obtain a baseline value. Each duplicate tube contained 1.6 mg pre-incubated synaptosomal protein obtained from rat brain (Lazarus et al., 1989a) in equilibrium assays containing 50 mM HEPES, pH 7.5, 5 mM $MgCl_2$, glycerol and protease inhibitors (Lazarus et al., 1989a, 1993) for 120 min at room temperature (22–23 °C). The membranes were trapped in the filters and rapidly washed within 5 sec with 3 × 2 mL ice-cold buffer containing BSA (Lazarus et al., 1989a). The labelled peptides were displaced using 6–14 concentrations of each analogue; the displacement curve ranged over a thousand-fold in peptide concentration. Competitive inhibition constants (K_i) were derived from the IC_{50} values using the equation of Cheng and Prusoff (1973).

Molecular modelling

Molecular dynamics performed on deltorphin A utilized using HyperChem™ (V. 3.0, Auto-Desk, Sausalito, CA) according to Bryant et al. (1993) on a Dell 450DE PC operating at 50 MHz. Starting structures were generated using dihedral angles from nuclear Overhauser

spectroscopy data calculated by Amodeo et al. (1992). Additional starting structures were generated to address the uncertainties (Nikiforovich et al., 1993) of the NMR data; i.e., the dihedral angles were varied $\pm 10°$, $\pm 20°$, and $\pm 30°$ (Bryant et al., 1993). Solvated low energy structures were generated under periodic boundary conditions by optimization of initial conformers in equilibrated TIP3P water molecules. Analyses were completed using the all-atom parametrization of AMBER force field with distant dependent dielectric (ε) for initial structures and constant dielectric ($\varepsilon = 1$) for solvated structures. Geometry optimizations were performed on each structure before and after solvation and incorporated the Polak Ribiere algorithm stopping with root mean square gradient of 10^{-2} Kcal/mol-Å or less. The conformational search for a global minima utilized a simulated annealing schema according to Tancredi et al. (1991) and generated more than 6,000 conformers per structure. The analysis employed six temperatures ranging from 1000 K to 10 K with time steps of 0.2 fs at 1000 K, 500 K, and 250 K, and 0.5 fs at 100 K, 50 K, and 10 K. The lowest energy conformers were chosen from snapshots taken at every time step, optimized and then used in the next annealing cycle.

Results

The importance of the imidazole side-chain at position 4 of the δ selective opioid peptide deltorphin A (H-Tyr-D-Met-Phe-His-Leu-Met-Asp-NH_2) on the receptor binding was investigated through a series of His[4]-modified and residue 4 replacement analogues; the substituents at position 4 and the protective groups on Asp[7], when applicable, are shown in Fig. 2.

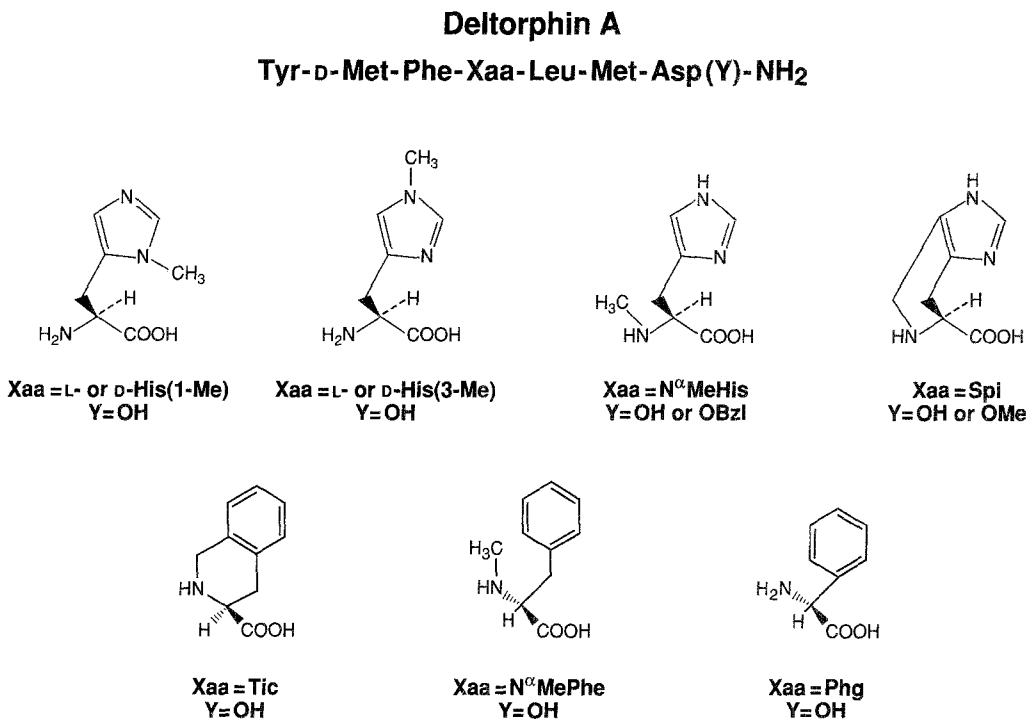

Fig. 2. Amino acid replacement residues at His[4] in deltorphin A and modification of the carboxy function of Asp[7]. Xaa represents His or the substituted residue at position 4. Y is either -OH or as the carboxyl protective ester -OBzl or -OMe on Asp[7]

Table 2. Receptor binding properties of His[4]-substituted deltorphin A*

No.	Peptide	$K_i\delta$	$K_i\mu$	$K_i\mu/K_i\delta$
1.	H-Tyr-D-Met-Phe-His-Leu-Met-Asp-NH_2	0.40 ± 0.06 (10)	223 ± 47 (8)	558
2.	H-Tyr-D-Met-Phe-His(1-Me)-Leu-Met-Asp-NH_2	0.38 ± 0.09 (4)	646 ± 74 (4)	950
3.	H-Tyr-D-Met-Phe-D-His(1-Me)-Leu-Met-Asp-NH_2	6.80 ± 1.47 (3)	2,030 ± 241 (4)	304
4.	H-Tyr-D-Met-Phe-His(3-Me)-Leu-Met-Asp-NH_2	0.74 ± 0.14 (4)	551 ± 105 (4)	1,147
5.	H-Tyr-D-Met-Phe-D-His(3-Me)-Leu-Met-Asp-NH_2	6.84 ± 2.14 (3)	6,565 ± 884 (4)	808
6.	H-Tyr-D-Met-Phe-N^{α}MeHis-Leu-Met-Asp-NH_2	3.37 ± 0.57 (3)	41.2 ± 5.78 (3)	12
7.	H-Tyr-D-Met-Phe-N^{α}MeHis-Leu-Met-Asp(OBzl)-NH_2	4.48 ± 0.92 (4)	2.63 ± 0.53 (3)	0.6
8.	H-Tyr-D-Met-Phe-Spi-Leu-Met-Asp-NH_2	0.57 ± 0.11 (3)	52.6 ± 10.9 (3)	92
9.	H-Tyr-D-Met-Phe-Spi-Leu-Met-Asp(OMe)-NH_2	0.99 ± 0.16 (3)	20.5 ± 2.45 (3)	21
10.	H-Tyr-D-Met-Phe-N^{α}MePhe-Leu-Met-Asp-NH_2	2.00 ± 0.36 (3)	14.3 ± 1.88 (3)	7
11.	H-Tyr-D-Met-Phe-Tic-Leu-Met-Asp-NH_2	0.76 ± 0.17 (4)	159 ± 26 (3)	209
12.	H-Tyr-D-Met-Phe-Phg-Leu-Met-Asp-NH_2	7.66 ± 1.29 (4)	393 ± 82 (5)	51

* The inhibition constants (K_i) for δ and μ receptors are given in nM; the number of repetitions are given in parentheses. Selectivity for the δ receptor is the ratio K_i^{μ}/K_i^{δ}.

Alkylation of the nitrogen groups on the imidazole side-chain (compounds **2–5**) produced minor changes in δ or μ affinities (Table 2), such that δ selectivity rose approximately 2-fold relative to deltorphin A (**1**) in analogues containing L-His[4]. The most disruptive modification for δ binding was the substitution by a D-enantiomer of the methylated His[4] residue (compounds **3** and **5**); δ and μ affinities fell more than an order of magnitude, although the high δ selectivity was only lost in one analogue, [D-His(1-Me)[4]]deltorphin A (compound **3**).

Constraints on the backbone (peptides **6–10**) substantially enhanced μ binding while exhibiting variable effects on δ affinities ranging from essentially no change (compound **8**) to losses of about 10-fold (analogues **6** and **7**) (Table 2). Interestingly, protection of the carboxyl function of Asp[7] with a benzyl group in the analogue [N$^{\alpha}$MeHis[4],Asp(OBzl)[7]] deltorphin A (peptide **7**) completely abolished all selectivity due to a near equivalency in the binding values for both δ and μ receptor sites. Cyclization of the amino group to the imidazole side-chain of His[4] to yield Spi[4]-derivatives (Fig. 2) (compounds **8** and **9**) or to the benzyl side-chain of Phe to form Tic (compound **11**) resulted in peptides which exhibited minimal effects on δ affinity while μ binding increased and, as a consequence, δ selectivity was substantially lost. Elimination of the carboxy function of Asp[7] in [Spi[4],Asp(OMe)[7]]deltorphin A (compound **9**) further increased μ binding.

Replacement of the imidazole side-chain by an aromatic ring, either as the constrained Phe-derivative, N$^{\alpha}$MePhe[4] (compound **10**), or a residue whose side-chain was spatially abbreviated (Phg) (peptide **12**) had opposite effects on receptor binding: N$^{\alpha}$Phe[4] decreased δ affinity 5-fold and increased μ binding 17-fold, while Phg[4] decreased δ and μ binding by 19-fold and roughly twice,

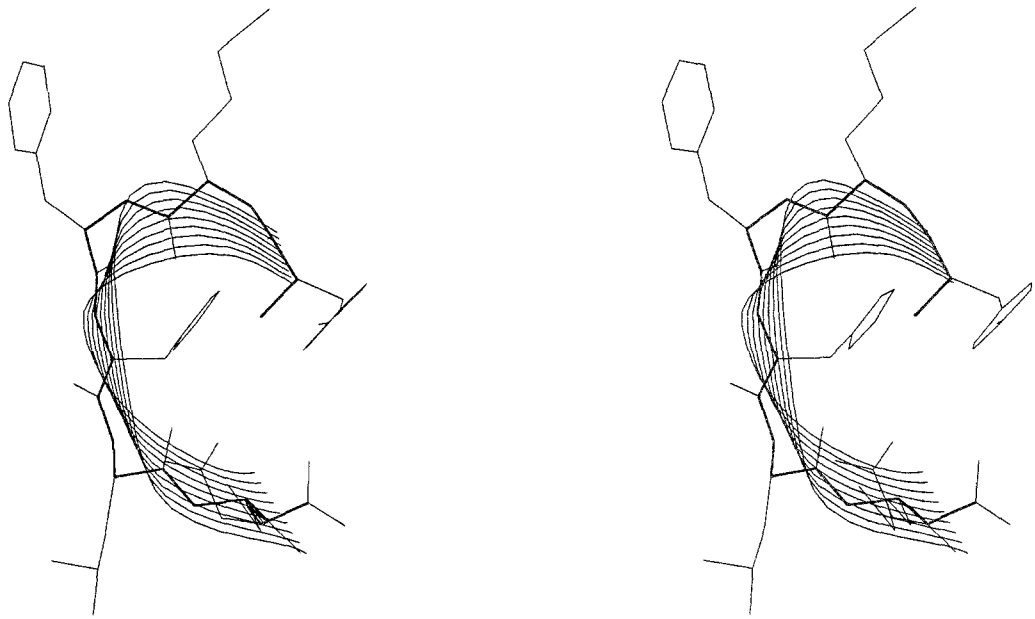

Fig. 3. Wall-eyed stereoview of a low energy conformer of deltorphin A

respectively. Thus, whereas δ selectivity was nearly eliminated in compound **10**, it decreased only about 80% in analogue **12** (Table 2), which was less than half of that seen with Phe^4 (Lazarus et al., 1993).

Molecular dynamics simulations revealed low energy conformers of deltorphin A (Fig. 3) where the N-terminal tripeptide prescribed a type II′ β-turn and the imidazole side-chain of His^4 was spatially oriented parallel to and opposite the phenolic ring of Tyr^1. These conformers indicated that the side-chain of D-Met^2 is positioned on the same side of the peptide as Tyr^1 and Phe^3. Furthermore, an additional β-turn is seen in the C-terminal region (denoted by the superimposed ribbon in Fig. 3).

Discussion

Alterations of the peptide backbone of deltorphin A at position 4 are less deleterious than comparable modifications at position 3 (Salvadori et al., 1993) on δ affinity. It appears that the major contributor to a disruption in δ affinity occurred as a result of reorientation of the imidazole side-chain due to a change in chirality (Table 2) (Lazarus et al., 1992) or the presence of either an anionic (Sagan et al., 1992; Lazarus et al., 1993) or cationic function (Salvadori et al., 1992; Sagan et al., 1992); residues containing aromatic side-chains generally had a less pronounced effect on δ affinity than on μ binding (Salvadori et al., 1992; Lazarus et al., 1993). However, in each case, δ selectivity was seriously impaired (Salvadori et al., 1992; Sagan et al., 1992; Lazarus et al., 1993). The structurally constrained position 4 analogues containing either Spi or Tic failed to affect δ affinity, although high δ selectivity was lost due to enhanced μ binding. It would appear that the ring structure of Spi or Tic imparts a limited degree of rigidity to the backbone of the peptide at position 4 which partially mimics a preferred conformation for recognition by the μ receptor.

The fact that alkylation of the nitrogens at position 1 or 3 of the imidazole ring of His^4 exhibited a minimal effect on δ affinity coupled to a substantial attenuation of μ binding leads us to conclude that a degree of latitude exists in the δ binding site of a bulkier side-chain vis-à-vis the constrained Spi^4 and Tic^4 (compounds **8** and **11**). Interestingly, the hydrophobic methyl groups increased δ selectivity by exerting an inhibition of binding to the μ receptor.

The global influence of the side-chain of His^4, nonetheless, seems to play a role in binding to δ receptors. For example, the aromatic side-chain of Phe^4, Tyr^4 (Salvadori et al., 1992; Lazarus et al., 1993) and N$^\alpha$MePhe^4 (Table 2) were less disruptive on δ affinity than Phg^4 which reduced δ affinity relative to the parent molecule (compound **1**). The structural difference between Phg and Phe is the loss of a -CH_2 group between the backbone and the side-chain, which reduces the projection of the benzyl side-chain by approximately 1.5 Å into a receptor pocket. This would appear to modify the conformation or spatial orientation of adjacent residues; however, Phg^4 also has the ability to modify the ϕ and ψ angles of the backbone environment. On the other hand, a Gly^4 substitution, which minimally effected δ affinity (Salvadori et al., 1992), indicates that perhaps the residue at position 4 plays a role in maintaining the proper intramolecular distance between the N- and C-terminal tripeptide sequences, as

suggested previously (Lazarus et al., 1992a) and might represent a separation of the "message" and "address" domains (Portoghese, 1989, 1993). However, the absence of a chiral center or a side-chain on Gly[4] resulted in higher μ binding (Salvadori et al., 1991). A glycine residue at position 4, moreover, may allow the solvated peptide to undergo more extensive long range interactions that might induce folded conformations (Premilat and Maigret, 1977) in which the hydrophobic regions become more favorable or accessible for binding to μ receptors. Furthermore, des-His[4] analogues which place the essential Leu[5] residue in an unfavorable location relative to the hydrophobic site in the receptor, exhibited weak δ binding and reduced selectivity without effecting μ affinity (Lazarus et al., 1992a); the correct positioning of a hydrophobic side-chain at position 5 is necessary for high δ affinity and selectivity (Sagan et al., 1989; Sasaki et al., 1991; Lazarus et al., 1992a,b, 1993).

The enhancement of μ binding properties in the presence of hydrophobic protecting groups on the carboxyl function of Asp[7] (compounds **7** and **9**) further implies a major difference between the δ and μ receptor in the apparent requirement for additional lipophilic centers in the opioid peptide. This observation supports the notion that the μ receptor prefers peptides with an increased number of hydrophobic side-chains and substituents (Lazarus et al., 1990). In fact, the elevation in μ binding, which occurred in all His[4] analogues containing a constrained backbone or side-chain (compounds **6–9**), reveals increased hydrophobicity as denoted by the higher capacity factors (Table 1).

The low energy conformers of deltorphin A from molecular dynamics simulations suggest that the relationship of the spatial orientation of the side-chains of His[4] and Tyr[1] approximates an optimal fit of this particular opioid peptide in the δ receptor; this conformation apparently hinders the peptide from interacting with the μ receptor. One might speculate, therefore, that this particular orientation of His[4] in deltorphin A facilitates the formation of β-turns in both the N-terminal and C-terminal portions of the peptide (Fig. 3); this supports NMR data which indicated that deltorphin A featured a succession of β-turns (Tancredi et al., 1991; Amodeo et al., 1992). Interestingly, the conformation of deltorphin A superficially resembles the S-shaped backbone described for deltorphin B (Ohno et al., 1993). The positioning of D-Met[2] in our model is consistent with other studies on deltorphin A (Nikiforovich and Hruby, 1990; Nikiforovich et al., 1991, 1993; Misicka et al., 1991; Tancredi et al., 1991; Amodeo et al., 1992; Bryant et al., 1993) in which Tyr[1] and Phe[3] are in a *trans* conformation (Yamazaki et al., 1993) and the side-chain of D-Xaa[2] is "sandwiched between the aromatic rings of Tyr[1] and Phe[3]" in peptides containing the common N-terminal tripeptide, H-Tyr-D-Xaa-Phe (Brantl et al., 1993). However, this particular low energy model differs from those presented by others in which the His[4] side-chain spatially occupies a region *trans* to Tyr[1] (Nikiforovich et al., 1991, 1993).

Disruption of the backbone dihedral angles or replacement of the side-chain at residue 4 could cause significant distortion of the peptide (Salvadori et al., 1993) which would allow greater interaction at the μ receptor site (*supra vide*). Nonetheless, the principle conclusions that can be drawn from this study are two-fold: (1) the imidazole side-chain of His[4] is responsible for maintaining the integrity of deltorphin A in an optimized conformation recognized by the δ

receptor site and one that simultaneously permits the peptide to resist binding to a μ receptor site; and (2) with at least one series of low energy conformers of deltorphin A, it appears highly probable that the projections of the side-chains of Tyr^1 and His^4 spatially exist parallel and opposite to each other in a structure that favors a series of β-turns.

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References

Amodeo P, Motta A, Tancredi T, Salvadori S, Tomatis R, Picone D, Saviano G, Temussi PA (1992) Solution structure of deltorphin I at 265: a quantitative NMR study. Peptide Res 5: 48–55

Brantl V, Picone D, Amodeo P, Temussi PA (1993) Solution structure of casokefamide. Biochem Biophys Res Commun 191: 853–858

Bryant SB, Salvadori S, Attila, M, Lazarus LH (1993) Topographical conformations of the deltorphin predicate delta opioid receptor affinity. J Am Chem Soc 115: 8503–8504

Chen, Y, Mestek A, Liu J, Hurley JA, Yu L (1993) Molecular cloning and functional expression of mu-like opioid receptor from rat brain. Mol Pharmacol 44: 8–12

Cheng YC, Prusoff WH (1973) Relationships between the inhibition constant (K_i) and the concentration of inhibition which causes 50 per cent inhibition (I_{50}) of an enzymatic reaction. Biochem Pharmacol 22: 3099–3108

Daly JW, Myers CW, Whittaker N (1987) Further classification of skin alkaloids from neotropical poison frogs (Dendrobatidae), with a general survey of toxic/noxious substances in the amphibia. Toxicon 25: 1023–1095

Erspamer V (1984) Half a century of comparative research on biogenic amines and active peptides in amphibian skin and molluscan tissues. Comp Biochem Physiol 79C: 1–7

Erspamer V (1992) The opioid peptides of the amphibian skin. Int J Devel Neurosci 10: 3–30

Erspamer V, Melchiorri P, Falconieri Erspamer G, Negri L, Corsi R, Severini C, Barra D, Simmaco M, Kreil G (1989) Deltorphins: a family of naturally occurring peptides with high affinity and selectivity for δ opioid binding sites. Proc Natl Acad Sci USA 86: 5188–5192

Evans CJ, Keith Jr DE, Morrison H, Magendzo K, Edwards RH (1992) Cloning of a delta opioid receptor by functional expression. Science 258: 1952–1955

Fukuda K, Kato S, Mori K, Nishi M, Takeshima H (1993) Primary structures and expression from cDNAs of rat opioid receptor δ- and μ-subtypes. FEBS Lett 327: 311–314

Kieffer BL, Befort K, Gaveriaux-Ruff C, Hirth CG (1992) The δ-opioid receptor: isolation of a cDNA by expression cloning and pharmacological characterization. Proc Natl Acad Sci USA 89: 12048–12052

Kreil G, Barra D, Simmaco M, Erspamer V, Falconieri Erspamer G, Negri L, Severini C, Corsi R, Melchiorri P (1989) Deltorphin, a novel amphibian skin peptide with high selectivity and affinity for δ opioid receptors. Eur J Pharmacol 162: 123–128

Lazarus LH, Attila M (1993) The toad, ugly and venomous, wears yet a precious jewel in his skin. Prog Neurobiol 41: 473–507

Lazarus LH, de Castiglione R, Guglietta A, Wilson WE (1989a) Dermorphin gene sequence peptide with high affinity for δ-opioid receptors. J Biol Chem 264: 3047–3050

Lazarus LH, Guglietta A, Wilson WE, Irons B, de Castiglione R (1989b) Dimeric der-

morphin analogues as μ-receptor probes on rat brain membranes: correlation between central μ-receptor potency and suppression of gastric acid secretion. J Biol Chem 264: 354–362

Lazarus LH, Wilson WE, Guglietta A, de Castiglione R (1990) Dermorphin interaction with rat brain opioid receptors: involvement of hydrophobic sites in the binding domain. Mol Pharm 37: 886–892

Lazarus LH, Salvadori S, Santagada S, Tomatis R, Wilson WE (1991) Function of negative charge in the "address domain" of deltorphins. J Med Chem 34: 1350–1355

Lazarus LH, Salvadori S, Balboni G, Tomatis R, Wilson WE (1992a) Stereospecificity of amino acid side chains in deltorphin defines binding to opioid receptors. J Med Chem 35: 1222–1227

Lazarus LH, Salvadori S, Grieco P, Wilson WE, Tomatis R (1992b) Unique sequence in deltorphin C confers structural requirements for δ opioid receptor selectivity. Eur J Med Chem 27: 791–797

Lazarus LH, Salvadori S, Attila A, Grieco P, Bundy DM, Wilson WE, Tomatis R (1993) Interaction of deltorphin with opioid receptors: molecular determinants for affinity and selectivity. Peptides 14: 21–28

Misicka A, Lipkowski AW, Fang L, Knapp RJ, Davis P, Kramer T, Burks TF, Yamamura HI, Carr DB, Hruby VJ (1991) Topographical requirements for delta opioid ligands: presence of a carboxyl group in position 4 is not critical for deltorphin high delta receptor affinity and analgesic activity. Biochem Biophys Res Commun 180: 1290–1297

Montecucchi PC, de Castiglione R, Piani S, Gozzini L, Erspamer V (1981a) Amino acid composition and sequence of dermorphin. A novel opiate-like peptide from the skin of *Phyllomedusa sauvagei*. Int J Pept Prot Res 17: 275–283

Montecucchi PC, de Castiglione R, Erspamer V (1981b) Identification of dermorphin and Hyp[6]-dermorphin in skin extracts of the Brazilian frog *Phyllomedusa rohdei*. Int J Pept Prot Res 17: 316–321

Mor A, Delfour A, Sagan S, Amiche M, Pradelles J, Rossier J, Nicolas P (1989) Isolation of dermenkephalin from amphibian skin, a high-affinity δ-selective opioid heptapeptide containing a D-amino acid residue. FEBS Lett 255: 269–274

Nikiforovich GV, Hruby VJ (1990) Examination of the conformational meaning of "δ-address" in the dermenkephalin sequence. Biochem Biophys Res Commun 173: 521–527

Nikiforovich GV, Hruby VJ, Prakash O, Gehrig CA (1991) Topographical requirements for δ-selective opioid peptides. Biopolymers 31: 941–955

Nikiforovich GV, Prakash O, Gehrig CA, Hruby VJ (1993) Conformations of the dermenkephalin backbone in DMSO solution by a new approach to the solution conformations of flexible peptides. J Am Chem Soc 115: 3399–3406

Ohno Y, Segawa M, Ohishi H, Doi M, Kitamura K, Ishida T, Inoue M, Iwashita T (1993) Conformation of deltorphin-II in membrane environment studies by two-dimensional NMR spectroscopy and molecular dynamics calculations. Eur J Biochem 212: 185–191

Pattabiraman N, Sorensen KR, Langridge R, Bhatnagar S, Renugopalakrishnan V, Rapaka RS (1986) Molecular mechanics studies of dermorphin. Biochem Biophys Res Commun 140: 341–349

Portoghese PS (1989) Bivalent ligands and the message-address concept in the design of selective opioid receptor antagonists. Trends Pharmacol Sci 10: 230–235

Portoghese PS (1993) The design of δ-selective opioid receptor antagonists. Farmaco 48: 243–251

Premilat S, Maigret B (1977) Effects of long range interactions on the conformational statistics of short polypeptide chains generated by a Monte Carlo method. J Chem Phys 66: 3418–3425

Richter K, Egger R, Kreil G (1987) D-Alanine in the frog skin peptide dermorphin is derived from L-alanine in the precursor. Science 238: 200–202

Richter K, Egger R, Negri L, Corsi R, Severini C, Kreil G (1990) cDNAs encoding (D-Ala[2])-deltorphin precursors from skin of *Phyllomedusa bicolor* also contain genetic information for three dermorphin-related opioid peptides. Proc Natl Acad Sci USA 87: 4836–4839

Sagan S, Amiche M, Delfour A, Mor A, Camus A, Nicolas P (1989) Differential contribution of C-terminal regions of dermorphin and dermenkephalin to opioid-sites selection and binding potency. Biochem Biophys Res Commun 163: 726–732

Sagan S, Charpentier S, Delfour A, Amiche A, Nicolas N (1992) The aspartic acid in deltorphin I and dermenkephalin promotes targeting to δ-opioid receptor independently of receptor binding. Biochem Biophys Res Commun 187: 1203–1210

Salvadori S, Marastoni M, Balboni G, Borea PA, Morari M, Tomatis R (1991) Synthesis and structure-activity relationships of deltorphin analogues. J Med Chem 34: 1656–1661

Salvadori S, Bianchi C, Lazarus LH, Scaranari V, Attila M, Tomatis R (1992) Para-substituted Phe[3] deltorphin analogues: enhanced selectivity of halogenated derivatives for δ opioid receptor sites. J Med Chem 35: 4651–4657

Salvadori S, Bryant SD, Bianchi C, Balboni G, Scaranari V, Attila M, Lazarus LH (1993) Phe[3]-Substituted analogues of deltorphin C. Spatial conformation and topography of the aromatic ring in peptide recognition by δ opioid receptors. J Med Chem 36: 3748–3756

Sasaki Y, Ambo A, Suzuki K (1991) [D-Ala[2]]Deltorphin II analogs with high affinity and selectivity for delta-opioid receptor. Biochem Biophys Res Commun 180: 822–827

Schiller PW, Nguyen TMD, Weltrowska G, Lemieux C, Chung NN (1990) Development of [D-Ala[2]]deltorphin I analogs with extraordinary delta receptor selectivity. In: van Ress JM, Mulder AH, Wigant VM, van Wimmersmagreidanus TB (eds) New leads in opioid research. Proceedings of the International Narcotics Research Conference, Excerpta Medica International Congress Series, vol 194. Excerpta Medica, Amsterdam, pp 288–290

Tancredi T, Temussi PA, Picone D, Amodeo P, Tomatis R, Salvadori S, Marastoni M, Santagada V, Balboni G (1991) New insights on μ/δ selectivity of opioid peptides: conformational analysis of deltorphin analogues. Biopolymers 31: 751–760

Yamazaki T, Ro S, Goodman M, Chung NN, Schiller PW (1993) A topographical approach to explain morphiceptin bioactivity. J Med Chem 36: 708–719

Yasuda K, Raynor K, Kong H, Breder CD, Takeda J, Reisine T, Bell GI (1993) Cloning and function comparison of κ and δ opioid receptors from mouse brain. Proc Natl Acad Sci USA 90: 6736–6740

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