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Structure-activity relationships of the δ-opioid-selective agonists, deltorphins

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Deltorphins are naturally occurring peptides with high affinity and selectivity for δ-opioid receptors. They share with dermorphin, another μ-selective opioid agonist, the same N-terminal tripeptide Tyr-D-Xaa-Phe, where D-Xaa is a D-Ala or a D-Met residue. This common sequence appears to be essential for the best fitting of the peptides to both μ- or δ-opioid sites. We studied the changes in receptor affinity and selectivity and in biological potency of deltorphins due to shortening of the sequence, C-terminal deamidation or single amino acid substitutions. The results support the view that a code addressing the molecule towards δ-opioid sites is expressed in the C-terminal region of these peptides. This addressing domain confers high δ-selectivity to the ligand in the following two ways: (i) increased affinity for δ-sites; (ii) decreased affinity for μ-sites. The sequence of the C-terminal tripeptide appears to be responsible for the high δ-affinity of the molecules. Negatively charged side chains inhibit μ-binding and enhance δ-selectivity.

Deltorphins; Synthetic peptides; Receptor binding assay; Vas deferens assay (mouse); δ-Opioid receptors (selectivity for)

1. Introduction

In earlier publications (Kreil et al., 1989; Erspamer et al., 1989), we described the isolation, synthesis and biological activity of a new family of naturally occurring heptapeptides endowed with high affinity and selectivity for δ-opioid receptors. These peptides have been named deltorphin, [D-Ala^2]deltorphin I and [D-Ala^2]deltorphin II and have in common the N-terminal sequence Tyr-D-Xaa-Phe, where D-Xaa is D-methionine in deltorphin and D-alanine in [D-Ala^2]deltorphins. The same N-terminal sequence is also present in dermorphin (Broccardo et al., 1981), an opioid peptide that binds with high affinity to μ-receptors and differs from deltorphins in the structure of the C-terminal tetrapeptide. We therefore argued that the C-terminal region of these peptides must be the address domain that is essential for opioid receptor selectivity (Erspamer et al., 1989). To study further the structural determinants of the high-affinity δ-binding of deltorphins, we have synthesized an extensive series of analogs and shorter homologues of deltorphins and demorphin. Like their parent compounds, these synthetic peptides contain the sequence Tyr-D-Xaa-Phe in their N-terminal region but have a different C-terminal structure. We now describe the in vitro bioassays for opioid activity and receptor binding properties of a number of these peptides. By comparing the data obtained with deltorphin analogs with the data on dermorphin analogs, reported now and in a previous study (De Castiglione and Rossi, 1985; De Castiglione et al., 1981; Melchiorri et al., 1982; Lazarus et al., 1989), we further discuss the structural and conformational features that determine the selectivity and affinity of these opioids towards the μ- and δ-types of receptors.

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2. Materials and methods

2.1. Peptide synthesis

Peptides were prepared by solid-phase synthesis on a Biolynx automated peptide synthesizer (Pharmacia Biochrom) according to fluoren-9-ylmethoxycarbonyl (Fmoc)-polyamide active ester chemistry (Atherton et al., 1975). The products were purified by preparative HPLC on a Zorbax Protein Plus column (21.2 × 250 mm; 10 μm particle size; 300 Å pore diameter; C3

packing; Dupont) with a linear gradient from 10 to 90% of solvent B (5 mM trifluoroacetic acid in acetonitrile) in solvent A (5 mM trifluoroacetic acid in water) over 60 min. Amino acid analyses after 6 M HCl hydrolysis (24 h, 110°C, in vacuo) gave ratios consistent with the desired structures. The expected amino acid sequences were confirmed by automated Edman degeneration performed with an Applied Biosystems 470A protein sequencer.

2.2. *Binding assays*

Binding of the peptides of μ- and δ-opioid sites was assayed in crude membrane preparations (Kosterlitz et al., 1980) from rat brain at pH 7.4 in 50 mM Tris-HCl buffer. Each assay contained, in a final volume of 2 ml, the membrane preparation (0.8-1.0 mg of membrane proteins) and the tritiated ligand at the desired concentration with or without unlabeled ligand. The μ-binding site was selectively labeled with [^{3}H][D-Ala^2,MePhe^4,Glyol^5]enkephalin ([^{3}H]DAGO, Amersham, UK) (1 nM); the δ-binding site with ^{3}H-labeled S-S cyclized [D-penicillamine^2,D-penicillamine^5]enkephalin ([^{3}H]DPDPE, Amersham, UK) (3 nM). Non-specific binding was determined in the presence of 50 μM naloxone (S.A.L.A.R.S., Como, Italy) or 5 μM naltrindole (R.B.I., Natick, MA). No protease inhibitor was added to the incubation medium since recovery experiments had demonstrated that the D-amino acid residue in position 2 and the C-terminal amide protected the peptides from attack by hydrolyzing enzymes. After a 90-min incubation at 35°C the free ligand was separated from membrane-bound ligand by filtration over Millipore AP40 glass fibre filters (soaked in 0.1% bovine serum albumin incubation buffer, for 1 h) under reduced pressure; the filters were then washed three times with 5 ml of ice-cold buffer. The radioactivity was extracted in 10 ml of Kontrogel (Kontron, Milano, Italy) and measured in a liquid scintillation counter (Betamatic V, Kontron, Milano, Italy). Competition curves were determined in triplicate and were usually obtained over the concentration range of 10 pM to 120 μM. The concentration of the peptides was determined by quantitative amino acid analysis. The IC_{50} value, graphically determined as $(B/B_0) \times 100$ plotted against the log of peptide concentration, was expressed as the concentration of peptide required to displace 50% of the labeled ligand. The inhibition constant (K_i) of the various non-radioactive peptides was calculated from IC_{50} by means of the equation $K_i = IC_{50}/[(2L/L_0) + (L/K_D)-1]$, where L is the free radioligand concentration in equilibrium with IC_{50} of unlabeled ligand, L_0 is the free radioligand concentration in the absence of competing ligand and K_D is the equilibrium dissociation constant of the radioligand.

2.3. *Recovery of the peptides from rat brain membrane preparation*

To 30 ml of rat brain membranes prepared in Tris-HCl 50 mM at protein concentration of 10 mg/ml, 10 μg of each peptide was added and the homogenate was incubated under continous stirring at 37°C for 90 min. After centrifugation the supernatant was passed through a C18 SEP-PAK cartridge (Waters Associates, Milford, MA, U.S.A.), washed three times with 5 ml of water and eluted with 2 ml of methanol. The eluates were evaporated to dryness in conical tubes by centrifugal force in vacuo (Savant Speed Vac Concentrator, Model SVC-100H, Savant Instruments Inc., New York, U.S.A.), then taken up in 100 μl of 0.2% TFA in water and injected into a reversed phase C18 HPLC column (4 × 250 mm, 5 μm particle size, 300 Å pore diameter, Dupont). Elution was carried out by a linear gradient of acetonitrile in 0.2% aqueous TFA, from 5 to 80%. An internal standard of [D-Ala^2]deltorphin I was added to the samples to calculate the recovery.

2.4. *Pharmacological assays in isolated tissues*

Preparations of the myenteric plexus-longitudinal muscle obtained from the small intestine of male guinea-pigs (GPI) (400-500 g) and preparations of mouse vas deferens (MVD) were used. The guinea-pig ileum was prepared as described by Gyang and Kosterlitz (1966) and mounted in an organ bath of 10 ml capacity. The tissues were bathed in the modified Krebs solution (mM) described by Kosterlitz et al. (1970): NaCl 118, KCl 4.75, $CaCl_2$ 2.54, KH_2PO_4 1.19, $MgSO_4$ 0.12, $NaHCO_3$ 25, glucose 11, gassed with 95% O_2 and 5% CO_2. The bath temperature was 37°C. The twitch-like contractions of the longitudinal muscle were recorded isometrically by a strain gauge transducer (DY 1, Basile, Milan) and displayed on a recording microdynamometer (Unirecord, Basile, Milan). The intramural nerves were stimulated with rectilinear pulse of 0.5 ms duration, given at intervals of 10 s using supramaximal voltage. The ileum was usually exposed to opioid peptides for 5 min until inhibition was maximal. The mouse vas deferens was prepared as described by Hughes et al. (1975). One pair, or less frequently two pairs, of vasa were mounted in an organ bath of 10 ml capacity. Longitudinal contractions were recorded as described for the guinea-pig ileum. The tissues were bathed in modified Krebs solution (mM): NaCl 118, KCl 4.75, $CaCl_2$, 2.54, $KHPO_4$ 0.93, $NaHCO_3$ 25, glucose 11, gassed with 95% O_2 and 5% CO_2. No protease inhibitor was added to the Krebs solutions. The bath temperature was 37°C. The intramural nerves were stimulated with trains of rectilinear pulses of 1 ms duration. Stimulation trains were given at intervals of 20 s and consisted of six stimuli of 1 ms duration with

TABLE 1

Changes in the inhibitory effects of demorphins due to shortening of the sequence and C-terminal deamidation. IC_{50} is the peptide concentration that produces 50% inhibition of contraction strength; MVD, mouse vas deferens preparation; GPI, guinea-pig ileum preparation; n, number of experiment; K_i, inhibition constant calculated from competitive binding curves; ala, D-alanine; met, D-methionine; n.d., not determined.

Peptides	IC_{50}, nM (mean ± S.E.)				MVD/GPI (IC_{50} ratio)	[^{3}H]DPDPE (K_i, nM)	[^{3}H]DAGO (K_i, nM)	δ/μ ($K_i\delta/K_i\mu$)
	MVD	n	GPI	n				
(1) Tyr-ala-Phe-Gly-Tyr-Pro-Ser-NH_2	16.2 ± 1.8	68	1.29 ± 0.09	85	12.56	185 ± 13	1.19 ± 0.07	155.0
(2) Tyr-ala-Phe-Gly-Tyr-Pro-NH_2	43.2 ± 3.9	10	2.73 ± 0.35	10	15.82	320 ± 19	1.60 ± 0.11	200.0
(3) Tyr-ala-Phe-Gly-Tyr-NH_2	23.8 ± 3.6	10	2.88 ± 0.37	9	8.26	88 ± 8.6	0.98 ± 0.11	97.8
(4) Tyr-ala-Phe-Gly-NH_2	263 ± 65	10	34.5 ± 4.71	10	7.62	1800 ± 180	11.9 ± 1.2	151.3
(5) Tyr-ala-Phe-NH_2	4050 ± 211	5	262 ± 30	5	15.46	n.d.	n.d.	n.d.
(6) Tyr-ala-Phe-Gly-Tyr-Pro-Ser-OH	28.1 ± 2.4	5	4.50 ± 0.32	5	6.24	n.d.	n.d.	n.d.

intervals of 10 ms. The results were expressed as the IC_{50} values obtained from concentration-response curves. When peptides were assayed, [D-Ala^2]deltorphin I and dermorphin were used as internal standards with mouse vas deferens and guinea-pig ileum preparations, respectively. Because preparations varied considerably in sensitivity, the results obtained for the various peptides were normalized as described by Kosterlitz et al. (1980). The IC_{50} value obtained in an individual assay was multiplied by the fraction: (mean IC_{50} value of the standard determined for all the assays)/(IC_{50} value of the standard found in the individual assay).

2.5. Data analysis

The accuracy of the determination of binding parameters was evaluated from the standard deviation of the error of the raw data, SD(E_{rad}) (Zivin and Waud, 1982). Analysis of variance (ANOVA) and multiple comparison tests (Scheffé, Fishers PLSD and Dunnett t) (Winer, 1971) were used to estimate the significance level of the observed differences in IC_{50} and K_i values of different peptides. Calculations were performed on a Macintosh II computer with the Stat View II (Abacus Concepts, Berkeley, CA) statistical program. To draw displacement curves, the data from ligand-binding experiments were analyzed by a weighted least-square curve-fitting algorithm essentially similar to that described by Munson and Rodbard (1980).

3. Results

3.1. Shorter homologues

As previously reported (Lazarus et al., 1989; Rossi et al., 1986), shortening of the dermorphin sequence from the C-terminal by one amino acid at a time produced a moderate decrease in the affinity for μ-receptors until the N-terminal tripeptide fragment was reached (tables 1, 2). Demorphin-(1-3) amide (table 1, No. 6) had virtually no affinity for either μ- or δ-binding sites, and dermorphin-(1-4) amide (table 1, No. 5) had a 10-fold lower affinity for μ-sites than dermorphin. Dermorphin-(1-5) amide and dermorphin-(1-6) amide (table 1, No. 3 and 2) had K_i for μ-sites that was close to that of dermorphin (table 1, No. 1). The selectivity of μ-sites however remained practically the same in the homologues with a chain length ranging from 4 to 6 amino acid residues (fig. 1). In the in vitro bioassays for opioid activity (table 1) the IC_{50} of these shorter dermorphin homologues confirmed the results of binding studies. The biological potency tended to diminish as the dermorphin sequence was shortened. In GPI preparations,

TABLE 2

Changes in the inhibitory effects of dermorphins due to single amino acid substitutions. IC_{50} is the peptide concentration that produces 50% inhibition of contraction strength; MVD, mouse vas deferens preparation; GPI, guinea-pig ileum preparation; n, number of experiments; K_i, inhibition constant calculated from competitive binding curves; ala, D-alanine; met, D-methionine; n.d., not determined.

Peptides	IC_{50}, nM (mean ± S.E.)				MVD/GPI (IC_{50} ratio)	[^{3}H]DPDPE (K_i, nM)	[^{3}H]DAGO (K_i, nM)	δ/μ ($K_i\delta/K_i\mu$)
	MVD	n	GPI	n				
(7) Tyr-met-Phe-Gly-Tyr-Pro-Ser-NH_2	249 ± 25	5	47 ± 5.1	5	5.3	1340 ± 162	92.4 ± 6.3	14.5
(8) Tyr-ala-Phe-Asn-Tyr-Pro-Ser-NH_2	30.2 ± 7.6	6	1.38 ± 0.15	6	21.88	235 ± 17	1.4 ± 0.05	174.0
(9) Tyr-ala-Phe-Ala-Tyr-Pro-Ser-NH_2	110 ± 8.1	5	14.4 ± 2.3	5	7.64	180 ± 22	4.6 ± 0.7	39.1
(10) Tyr-ala-Phe-His-Tyr-Pro-Ser-NH_2	603 ± 50	11	211 ± 45	6	2.86	2700 ± 230	55.0 ± 6.7	49.0
(11) Tyr-ala-Phe-Lys-Tyr-Pro-Ser-NH_2	2900 ± 340	6	2150 ± 157	7	1.35	9000 ± 1010	1037 ± 114	8.68
(12) Tyr-ala-Phe-Asp-Tyr-Pro-Ser-NH_2	1730 ± 191	11	821 ± 116	7	2.10	2941 ± 175	1746 ± 104	1.68
(13) DAGO	115 ± 21.2	10	7.1 ± 0.9	10	16.2	430 ± 58	1.36 ± 0.89	316.2

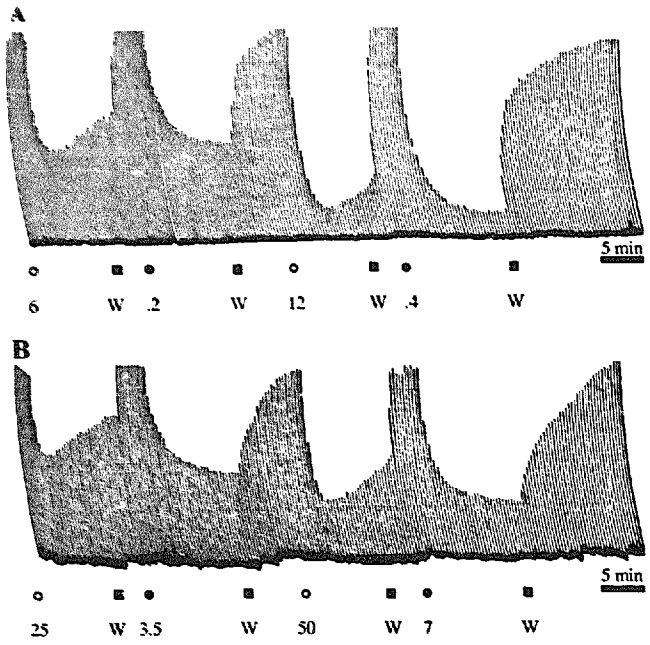

Fig. 1. Bioassay vas deferens preparation of [D-Ala^2]deltorphin I (A) and [D-Ala^2]deltorphin-(1-6) (B) with free (○) or amidated (●) C-terminal residue. W at (■) indicates washing. Numbers refer to peptide concentration in nM.

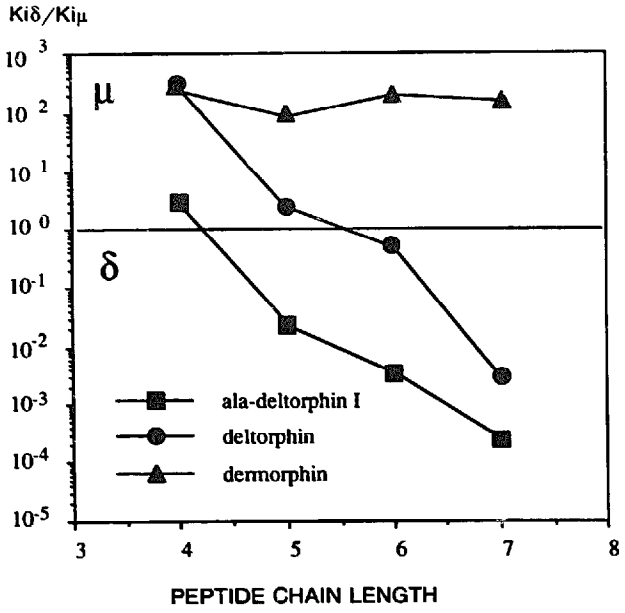

Fig. 2. Selectivity ($K_i\delta/K_{i\mu}$) of the shorter homologues of deltorphins and dermorphin relative to the number of amino acid residues. Non-selective ligands fall near the horizontal line at 10° selectively with δ-selective ligands below and μ-selective ligands above.

the N-terminal tetrapeptide had a 30-fold higher IC_{50} value than dermorphin.

Inhibition of δ-opioid receptor binding by [D-Ala^2]deltorphins and their shorter homologues produced a series of parallel displacement curves. When [D-Ala^2]deltorphin I (table 3, No. 1) was shortened from the C-terminus, the affinity for δ-opioid receptors decreased (table 3, peptides No. 2, 3 and 4). In vitro bioassays for δ-opioid activity carried out on mouse vas deferens preparations confirmed the loss of potency of the shorter homologues of [D-Ala^2]deltorphin I. [D-Ala^2]deltorphin I-(1-6)-amide showed an IC_{50} value 30-fold higher than that of the parent compound; [D-Ala^2]deltorphin I-(1-5) amide was about 300 times less potent than [D-Ala^2]deltorphin I and [D-Ala^2]deltorphin I-(1.4) amide was practically inactive. These results are further support for our previous proposal (Erspamer et al., 1989) that the C-terminal sequence of [D-Ala^2]deltorphins plays an address role in directing the peptides towards the δ-opioid receptors and are in agreement with results obtained by Sagan et al. (1989) with shorter homologues of DREK. DREK is the same peptide we isolated previously from amphibian skin and named deltorphin (Kreil et al., 1989). However, when the deltorphin and [D-Ala^2]deltorphin sequences were shortened, the affinity for μ-opioid receptors underwent different changes according to the C-terminus of the individual peptide. Shortening of the deltorphin sequence markedly increased the affinity towards μ-opioid receptors and the ratio $K_i(\delta)/K_i(\mu)$, whereas shortening of [D-Ala^2]deltorphin I produced a lower increase in the affinity for μ-sites and in the ratio $K_i(\delta)/K_i(\mu)$ (fig. 2). This difference appears to be crucial in the case

TABLE 3

Changes in the inhibitory effects of deltorphins due to shortening of the sequence and C-terminal deamidation. IC_{50} is the peptide concentration that produces 50% inhibition of contraction strength; MVD, mouse vas deferens preparation; GPI, guinea-pig ileum preparations; n, number of experiments; K_i, inhibition constant calculated from competitive binding curves; ala, D-alanine; met, D-methionine; n.d., not determined.

Peptides	IC_{50}, nM (mean ± S.E.)					MVD/GPI (IC_{50} ratio)	[^{3}H]DPDPE (K_i, nM)	[^{3}H]DAGO (K_i, nM)	δ/μ ($K_i\delta/K_i\mu$)
	MVD	n	GPI	n					
(1) Tyr-ala-Phe-Asp-Val-Val-Gly-NH_2	0.19 ± 0.01	98	1239 ± 203	17		1.5×10^{-4}	0.22 ± 0.05	1985 ± 224	1.1×10^{-4}
(2) Tyr-ala-Phe-Asp-Val-Val-NH_2	5.72 ± 0.43	10	501 ± 73	8		1.1×10^{-2}	1.07 ± 0.17	242 ± 37	4.4×10^{-3}
(3) Tyr-ala-Phe-Asp-Val-NH_2	60.2 ± 5.63	12	384 ± 106	7		1.6×10^{-1}	14.04 ± 1.35	619 ± 79.3	2.3×10^{-2}
(4) Tyr-ala-Phe-Asp-NH_2	> 2000	4	> 3000	4		n.d.	1254 ± 103	195 ± 31	6.43
(5) Tyr-ala-Phe-Asp-Val-Val-Gly-OH	5.32 ± 0.38	18	3280 ± 1114	7		1.6×10^{-3}	6.26 ± 0.89	1556 ± 309	4.0×10^{-3}
(6) Tyr-ala-Phe-Asp-Val-Val-OH	39.9 ± 2.90	12	2615 ± 340	4		1.5×10^{-2}	n.d.	n.d.	n.d.
(7) Tyr-ala-Phe-Asp-Val-OH	561 ± 74.1	6	2685 ± 176	4		2.1×10^{-1}	n.d.	n.d.	n.d.

of N-terminal tetrapeptide amides. Unlike the deltorphin homologue, Tyr-D-Met-Phe-His-NH_2, which behaves as a selective μ agonist ($K_i(\mu) = 8.0 \pm 0.4$ nM; $K_i(\delta)/K_i(\mu) = 167$), the [D-Ala^2]deltorphin I tretrapeptide, Tyr-D-Ala-Phe-Asp-NH_2, is devoid of biological activity and displays low affinity and selectivity toward μ-receptors ($K_i(\mu) = 195 \pm 51$ nM; $K_i(\delta)/K_i(\mu) = 6.4$). ANOVA and multiple comparison tests performed on IC_{50} values obtained with shortened analogs in the two bioassays gave the following results. MVD test: ANOVA, $F(2,117) = 537.15$, $P < 0.001$; Fisher PLSD, Scheffé F and Dunnett t significant at 95% for the comparison of IC_{50} values of the following pairs of peptides: 1 vs. 2, 1 vs. 3, 2 vs. 3 GPI test: ANOVA, $F(2,29) = 6.13$, $P = 0.006$; Fisher PLSD, Scheffé F and Dunnett t significant at 95% for the comparison of IC_{50} values of the following pairs of peptides: 1 vs. 2, 1 vs. 3. As experiments with substituted analogs confirm further, in the [D-Ala^2]-deltorphin homologues, the negatively charged side chain of Asp^4 or Glu^4 wastes the affinity for μ-binding sites. On this basis, the high δ-selectivity of [D-Ala^2]deltorphins may arise from a simultaneous increase in affinity at the δ-site and a decrease at the μ-site. Since increases selectivity can be attributed to a better fit at the target site in the first case only, apparent affinity should be regarded as the most meaningful parameter in the analysis of structure-activity relationships.

3.2. Deamidation of C-terminal amino acid

Deamidation of the C-terminal amino acid residue in both dermorphin and [D-Ala^2]deltorphin I, generated peptides with lower bioactivity and selectivity for the target receptors than the corresponding amides (table 1, No. 6; table 3, No. 5). Moreover, shortening of the sequence of deamidated [D-Ala^2]deltorphin I produced a loss of bioactivity on MVD with no significant parallel increase in the activity on GPI (table 3, No. 6, 7) (MVD test: ANOVA, $F(2,33)$]146.08, $P < 0.0001$; Fisher PLSD, Scheffé F and Dunnett t significant at 95% for peptides 5 vs. 7 and 6 vs. 7. GPI test: ANOVA, $F(2,12) = 0.13$, $P = 0.8766$). In the MVD preparation (fig. 1) deamidated analogs produced a more prompt depression of twitch and allowed a more rapid recovery after washing than did their parent amides. Moreover the twitch amplitude of MVD tended to recover spontaneously during exposure to deamidated peptides whereas it was further depressed by prolonging the contact with peptide amides. This behaviour may be partially explained by a more rapid hydrolysis of deamidated peptides than of the corresponding amides in the assay systems. The percent recovery of the peptide amides after 60 min incubation with brain membranes in Tris-HCl 50 mM at pH 7.4 ranged from 78 to 85% while the recovery of the deamidated peptides 5, 6, 7 (table 3) was 15, 32 and 64%, respectively.

The following variances were found for IC_{50} values obtained in the two bioassays for combined effects of shortening and C-terminal deamidation (two-factor ANOVA): for the main effect of peptide shortening in the MVD test, $F = 541.18$, $P < 0.001$ and in the GPI test, $F = 1.29$, $P = 0.2852$; for the main effect of deamidation of the C-terminal in the MVD test, $F = 512.83$, $P < 0.0001$ and in the GPI test $F = 20.19$ and $P < 0.0001$. For the interaction between shortening and deamidation, ANOVA yielded $F = 351.42$ and $P < 0.0001$ in the MVD test, and $F = 0.02$ and $P = 0.9844$ in the GPI test.

In order to elucidate better the interactions of side chains of ligands with the functional groups of δ-opioid receptors, we synthesized a number of substituted analogues.

3.3. Single amino acid substitution

The second amino acid residue in the common N-terminal sequence Tyr-D-Xaa-Phe is D-Met in del-

TABLE 4

Changes in the inhibitory effects of deltorphins due to single amino acid substitutions. IC_{50} is the peptide concentration that produces 50% inhibition of contraction strength; MVD, mouse vas deferens preparation; GPI, guinea-pig ileum preparation; n, number of experiments; K_i, inhibition constant calculated from competitive binding curves; ala, D-alanine; met, D-methionine; n.d., not determined.

Peptides	IC_{50}, nM (mean ± S.E.)				MVD/GPI (IC_{50} ratio)	[^{3}H]DPDPE (K_i, nM)	[^{3}H]DAGO (K_i, nM)	δ/μ ($K_i\delta/K_i\mu$)
	MVD	n	GPI	n				
(8) Tyr-met-Phe-His-Leu-Met-Asp-NH_2	0.97 ± 0.05	83	1476 ± 185	12	6.6×10^{-4}	1.18 ± 0.21	693 ± 37	1.7×10^{-3}
(9) Tyr-ala-Phe-His-Leu-Met-Asp-NH_2	1.62 ± 0.22	14	161 ± 25	8	1.0×10^{-2}	2.24 ± 0.31	358 ± 29	6.2×10^{-3}
(10) Tyr-met-Phe-Asp-Val-Val-Gly-NH_2	173 ± 40	8	1700 ± 390	4	1.0×10^{-1}	41.8 ± 5.90	3142 ± 175	1.3×10^{-2}
(11) Tyr-ala-Phe-Glu-Val-Val-Gly-NH_2	0.37 ± 0.02	44	2303 ± 415	10	1.6×10^{-4}	0.29 ± 0.07	2222 ± 233	1.3×10^{-4}
(12) Tyr-ala-Phe-Gly-Val-Val-Gly-NH_2	2.62 ± 0.32	14	22 ± 3	11	1.2×10^{-1}	3.26 ± 0.37	13.5 ± 2.1	2.4×10^{-1}
(13) Tyr-ala-Phe-Ala-Val-Val-Gly-NH_2	0.76 ± 0.08	10	91 ± 21	5	8.2×10^{-3}	0.66 ± 0.03	25.6 ± 2.7	2.6×10^{-2}
(14) Tyr-ala-Phe-Val-Val-Val-Gly-NH_2	1.53 ± 0.16	10	898 ± 188	4	1.7×10^{-3}	0.43 ± 0.05	40.0 ± 5.5	1.0×10^{-2}
(15) Tyr-ala-Phe-His-Val-Val-Gly-NH_2	0.83 ± 0.09	14	143 ± 27	10	5.9×10^{-3}	0.25 ± 0.02	83.0 ± 5.7	3.0×10^{-3}
(16) Tyr-ala-Phe-Asn-Val-Val-Gly-NH_2	4.45 ± 0.54	10	1630 ± 305	5	2.7×10^{-3}	1.16 ± 0.18	309 ± 19	3.7×10^{-3}
(17) Tyr-ala-Phe-Asp-Tyr-Val-Gly-NH_2	2.21 ± 0.18	10	1460 ± 200	5	1.5×10^{-3}	4.7 ± 0.59	3135 ± 297	1.5×10^{-3}
(18) DPDPE	6.05 ± 0.68	13	2698 ± 645	15	2.2×10^{-3}	8.5 ± 1.75	990 ± 98	8.6×10^{-3}

torphin and D-Ala in [D-Ala^2]deltorphins and dermorphin. In deltorphin, replacement of D-Ala for D-Met (table 4, No. 8, 9) produced minimal changes in δ-affinity, selectivity and biological potency. In contrast, in [D-Ala^2]deltorphin I, substitution of D-Met for D-Ala (table 4, No. 10) caused a decrease of two-three orders of magnitude in the apparent affinity for δ-sites and in the biological potency on mouse vas deferens. In dermorphin the same substitution produced a 100-fold fall in μ-affinity and biological potency (table 2, No. 7). Because the side chain of the amino acid residue in position 4 appears to be crucial to receptor type selectivity we studied the effects of changes in the charge and structure of this side chain on affinity, selectivity and biological potency. In dermorphin, substitution of Gly^4 with a negatively charged residue (Asp) produced an inactive peptide (table 2, No. 12), whereas substitution of Gly^4 with an uncharged residue to similar steric hindrance (Asn) (table 2, No. 8), gave an active peptide with affinity for μ-receptors and biological potency only slightly lower than that of dermorphin. In terms of δ-affinity and bioactivity, replacement of Asp^4 or Glu^4 by His, in [D-Ala^2]deltorphins (table 4, peptide No. 15) represents a neutral modification; μ-affinity, however, was enhanced and this led to a substantial drop in δ-selectivity. In addition to charge, changes in steric hindrance of the side chain in position 4 affect binding to μ-sites more than binding to δ-sites. The first member of this series of substituted analogs, [D-Ala^2, Gly^4]deltorphin (table 4, No. 12), is a peptide with an affinity for δ-receptors only 4 times greater than that for μ-receptors and consequently with very low selectivity. It behaves as a δ- and μ-agonist in in vitro bioassays for opioid activity. Substitution of Ala, Val, His or Asn for Asp^4 or Glu^4 (table 4, No. 13, 14, 15 and 16) produced peptides with K_i for δ-sites in a narrow range (0.3-1 nM) and K_i for μ-sites ranging from 25 to 309 nM. Thus δ-selectivity increases mainly because of a fall in μ-affinity. In the dermorphin sequence, substitution of Asn, Ala, His or Lys for Gly^4 (table 2, No. 8, 9, 10 and 11) generated peptides with K_i for μ-sites ranging from 1.4 (Asn) to 1037 nM (Lys).

ANOVA and multiple comparison tests performed on IC_{50} values obtained in the bioassays with these substituted analogs of deltorphins gave the following results. MVD test: ANOVA, $F(10,304) = 79.49$, $P < 0.0001$; PLSD (Fisher), F (Scheffé) and t (Dunnett) significant at 99% for the comparison of IC_{50} values of the following pairs of peptides: 1 vs. 10, 1 vs. 12, 1 vs. 14, 1 vs. 15, 1 vs. 16, 1 vs. 17; 11 vs. 12, 11 vs. 14, 11 vs. 16, 11 vs. 17; 12 vs. 13, 12 vs. 14, 12 vs. 15, 12 vs. 16, 12 vs. 17; 13 vs. 16; 14 vs. 16; 15 vs. 16 and 16 vs. 17. GPI test: ANOVA, $F(10,81) = 14.26$ $P < 0.0001$; PLSD (Fisher), F (Scheffé) and t (Dunnett) significant at 99% for the comparison of IC_{50} values of the following pairs of peptides: 1 vs. 10, 1 vs. 12, 1 vs. 15, 8 vs. 9, 11 vs. 12, 11 vs. 13, 11 vs. 15, 12 vs. 16.

4. Discussion

The emergence of the concept of multiple opioid receptors has led to numerous efforts aimed at developing highly selective ligands for the various receptor types (μ, δ, κ, etc.). Although various strategies have been used in the design of such specific ligands, compounds with high affinity for the desired receptor type are generally obtained by modifying naturally occurring peptides, through the classical approach of amino acid substitution, addition and deletion. We have recently succeeded in isolating from amphibian skin a new class of naturally occurring peptides with high affinity and selectively for δ-opioid receptors (Kreil et al., 1989; Erspamer et al., 1989). Due to their binding properties these peptides have been named deltorphin, [D-Ala^2]deltorphin I and [D-Ala^2]deltorphin II. They have in common the N-terminal sequence Tyr-D-Xaa-Phe, where D-Xaa is a D-Ala or D-Met, but differ as to the C-terminal tetrapeptide. However, all three deltorphins share a negatively charged amino acid residue in the C-terminal tetrapeptide, but deltorphin has it at the C-terminal (Asp^7-NH_2) while [D-Ala^2]deltorphin I and II contain an Asp or Glu residue in position 4.

We have shown here that shortening of the [D-Ala^2]deltorphin I sequence from the C-terminal produced a severe loss of affinity and selectivity for δ-sites and a sharp decrease in δ-specific biological activity. At first sight, these results appear similar to those obtained by Sagan et al. (1989) with shorter homologues of deltorphin. However shortening of the [D-Ala^2]deltorphin I sequence produced negatively charged peptides with a selectivity for δ-sites one order of magnitude greater than the uncharged deltorphin homologues of corresponding length. In deltorphin, cutting off the negatively charged Asp^7 residue produced a large drop in δ-selectivity by enhancing mainly μ-affinity. Using substituted analogs, we further demonstrated that negatively charged side chains (Asp and Glu) in the C-terminal sequence of [D-Ala^2]deltorphins enhance δ-selectivity by strongly inactivating the μ-binding of the molecule. This is presumably due to the increased electrostatic ligand repulsion by a negatively charged μ-receptor site or by a negatively charged membrane compartment that contains μ-receptors. However, since substitution of His or Asn for Glu^4 or Asp^4 in [D-Ala^2]deltorphins failed to produce important changes in K_i values for δ-sites, such electrostatic interactions between the peptide and the preferred receptor type or receptor-containing membrane compartment clearly played no role in the binding to δ-opioid sites. Dermorphin and

deltorphins are heptapeptide amides. Deamidation of the C-terminal amino acid residue was highly detrimental to δ-binding and δ-biological activity of deltorphins, but affected the μ-affinity and μ-biological potency of dermorphin to a lesser extent. Furthermore, demorphin-(1-6)-COOH retained 30-40% of the biological activity of demorphin, whereas all the shorter deamidated homologues of deltorphins showed heavy losses of biological activity in the MVD test. Thus, the biological activity of deamidated deltorphins might be deeply impaired in vitro and in vivo by carboxypeptidase shortening.

In conclusion, these results support the previous proposal (Erspamer et al., 1989) that a code addressing the molecule toward δ-opioid receptors is expressed in the C-terminal region of these peptides. This address domain confers high δ-selectivity to the ligand in the following two ways: (i) increased affinity for δ-sites; ii) decreased affinity for μ-sites. In this context, negatively charged side chains in the C-terminal region seem to have the function of taking the molecule away from μ-sites. Although we have not tested these synthetic peptides for binding to κ-opioid receptors and cannot exclude a priori κ-bioactivity to some extent, structural determinants for κ-affinity appear to differ greatly from those discussed here (Goldstein, 1988).

Finally, since [D-Ala^2]deltorphins are potent and selective δ-receptor probes, synthesis of analogs may provide useful tools for exploring the ill-defined physiological role and behavioural actions of δ-opioid receptors.

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