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Dermorphin Interaction with Rat Brain Opioid Receptors: Involvement of Hydrophobic Sites in the Binding Domain

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SUMMARY

Dermorphin structural analogues were utilized to determine the nature of opioid receptor subsite specificity, affinity, and selectivity in rat brain membranes. The data suggest that these parameters are influenced by the amino acid composition and sequence and the known solution conformation of dermorphin, in addition to the conformation of the membrane receptor. Two hydrophobic components of dermorphin are required for optimal binding. One component encompasses the stacked phenol groups in Tyr^1 and Tyr^5; the second involves the phenyl group of Phe^3. Evidence to support this proposal includes the following results: (a) removal of aromaticity, as occurs in [des-Tyr^5]- and [Gly^5]dermorphin, drastically reduced binding to both μ and δ sites; (b) inversion of the Phe^3-Gly^4 sequence in dermorphin to the Gly^3-Phe^4 in enkephalin enhanced binding to δ receptor sites, yet the peptide remained μ-selective; (c) substitution of Pro^4 for Gly^4 disrupted the solution conformation of dermorphin and decreased affinities at both receptor subsites, substantiating the requirement for the Phe^3-Gly^4-Tyr^5 sequence in dermorphin to interact with μ sites; and (d) modification of the serine residue, as occurs in [Ser(Bzl^7)] dermorphin and [Ser-NHNHZ^7]dermorphin, enhanced interaction with δ opioid receptors; the apparent δ affinity increased over 50-fold with [Ser(Bzl^7)]dermorphin, although it retained a weak μ-selectivity. However, both [Ser(Bzl^7)]- and [Ser-NHNHZ^7] dermorphin exhibited high affinity for μ receptor sites. Furthermore, the D-configuration about the α-carbon of residue 2 and the α-amine function and hydroxyl group on Tyr^1 are essential for receptor binding. We conclude that μ-opioid receptors contain distinct regions that accomodate the stacked phenol groups of Tyr^1 and Tyr^5 residues and the phenyl group of Phe^3.

In recent years, several amphibian heptapeptides containing a D-amino acid in position 2 were found to have very high affinities and selectivities for μ- and δ-, but not κ-, type mammalian opioid receptors (1–6). The dermorphins are capable of exhibiting high selectivity as μ-opioid agonists (1, 2, 7); an extensive series of synthetic peptide analogues has provided considerable information on the amino acid residues and sequences required for the opioid bioactivity of these peptides on pharmacological preparations (7–9). Conversely, the deltorphins [also called dermorphin gene-associated peptides (3) or dermenkephalins (10, 11)] (3–6, 10, 11) are very selective, high affinity ligands for the δ-receptor subtype, surpassing the enkephalin-derived agonists by several orders of magnitude (3–6). Deltorphin A, which is encoded by the dermorphin gene (12), contains D-Met^2 which occurs in a general *N*-terminal tripeptide sequence (Tyr^1-D-Xxx^2-Phe^3) similar to that in dermorphin (6). The dermorphins and deltorphins are potentially valuable in permitting further exploration of the microenvironment of mammalian opioid receptor systems involved in regulation of peripheral as well as central functions. The well known μ and δ receptor subtype characteristics of guinea pig ileum and mouse vas deferens, involving inhibition of electrically induced stimulation, were used to assess pharmacological properties of these two classes of D-amino acid-containing peptides (1, 2, 7–9, 11, 13–17). Further studies permitted delineation of specific residues required for binding of dermorphin to brain tissue membrane receptors (10, 13–16, 18, 19). Those results were particularly useful in permitting recognition of positive correlations between opioid receptor selectivities and the ability of dermorphin analogues to modulate physiological functions (8, 9, 18, 20).

This investigation on the influence of novel structural modifications of dermorphin on opioid receptor interactions provides additional insight into the molecular environment of μ-type receptor sites. The results are discussed in the context of NMR studies in the literature, which furnished information on the contribution of various functional groups and side chains of amino acid residues to the preferred (minimal energy) solution conformation of dermorphin and related peptides (21–30).

Experimental Procedures

Materials. Dermorphin and its analogues were synthesized by conventional segment condensation in solution; physiochemical properties and bioactivity spectra (smooth muscle contractility and analgesia)

ABBREVIATIONS: DAGO, [D-Ala^2,*N*-Me-Phe^4,Gly-ol]enkephalin; Boc, *tert*-butoxycarbonyl; BSA, bovine serum albumin; Bzl, benzyl; DADLE, [D-Ala^2,D-Leu^5]enkephalin; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (pK_a = 7.5); Z, benzyloxycarbonyl (carbobenzoxy).

were characterized as described previously (8, 9, 31). DAGO, DADLE, and [*N*-Me-Phe^3,D-Pro^4]morphiceptin were commercial products from Bachem (Torrance, CA) and Peninsula Laboratories (Belmont, CA). [^{3}H]DAGO (60.0 Ci/mmol) and [^{3}H]DADLE (44.1 Ci/mmol) were obtained from Amersham (Arlington Heights, IL). BSA (radioimmunoassay grade), bacitracin, bestatin, soybean trypsin inhibitor, and HEPES were purchased from Sigma Chemical Co. (St. Louis, MO). Glass microfiber filters (GF/C) were from Whatman International Ltd. (Maidstone, England). All other chemicals were of the highest purity available.

Peptide stock solutions (100 μg/ml) were prepared in either 50% ethanol or acidic alcohol (15% ethanol containing 10 mM acetic acid) and stored at 4°; dilutions were prepared weekly in polypropylene tubes, in 1 mg of BSA/ml of acidic alcohol, and kept at 4°. These conditions minimized absorptive losses of peptides on vessel walls for extended periods of time. Bacitracin and bestatin were aqueous solutions (10 mg/ml and 200 μM, respectively).

Synaptosome membranes. A synaptosomal fraction (P_2) was prepared, according to the method of Chang and Cuatrecasas (32), from the brains (minus cerebellum) of male Sprague-Dawley rats (Charles River, Raleigh, NC) in 10 volumes (w/v) of 0.32 M sucrose containing 10 mM HEPES, pH 7.5, and 50 μg/ml soybean trypsin inhibitor (3, 18). Cellular debris was sedimented by centrifugation at 3500 × *g* for 10 min at 4° and then the combined supernatants centrifuged at 40,000 × *g* for 20 min at 4°. The initial high speed membrane fraction was incubated, to eliminate endogenous opioid peptides, in 3 volumes (w/v) of a solution containing 50 mM HEPES, pH 7.4, 0.1 M NaCl, 50 μg/ml soybean trypsin inhibitor, and 0.1 mM GDP, for 60 min at 22–23° (3, 18). The synaptosome preparation was washed three times in buffer (50 mM HEPES, pH 7.5) containing 50 μg/ml soybean trypsin inhibitor, and the final washed membrane pellet was resuspended with a Teflon-glass homogenizer to give a concentration of 40 mg of protein/ml of storage solution (20% glycerol, 50 mM HEPES, pH 7.5, 50 μg/ml soybean trypsin inhibitor); 4-ml aliquots were stored at −70°. The presence of glycerol enabled the membrane fraction to be refrozen and used in subsequent binding studies without deleterious effects on the IC_{50} values (18); synaptosomes were generally thawed only once.

After solubilization of an aliquot of synaptosomes by a 1:10 dilution in 2% sodium dodecyl sulfate and boiling for 2 min in the presence of 5 mM 2-mercaptothanol, protein was estimated according to the method of Lowry *et al.* (33). Omission of the boiling step results in a nonlinear chromophore production relative to BSA.

Receptor binding assay. The μ and δ receptor binding assays were conducted in duplicate, in polypropylene tubes at 22–23° for 60 min, using 1.6 mg of membrane protein in a 1.0-ml volume assay containing 50 mM HEPES, pH 7.5, 8% glycerol, 100 μg of BSA, 20 μg of trypsin inhibitor, 1 mM $MgCl_2$, 1 μM bestatin, 25 μg of bacitracin, and radioligand; the concentrations of [^{3}H]DAGO and [^{3}H]DADLE were 0.68 ± 0.03 pmol and 0.87 ± 0.04 pmol in the μ and δ binding assays, respectively (3, 18). The specific μ agonist [*N*-Me-Phe^3,D-Pro^4]morphiceptin (34) (2.6 μM) was included in all δ binding assays to suppress binding to μ sites (3, 18); in its absence, binding to δ receptors was neither parallel to that of the δ peptide standard (DADLE) nor consistently reproducible. In the presence of morphiceptin, "[^{3}H]DADLE binds exclusively to δ receptors" (35; p.8). The radioreceptor assay mixtures were diluted with 1 ml of ice-cold buffer (20 mM HEPES, pH 7.5, containing 1 mg/ml BSA) and rapidly filtered (<5 sec) through presoaked (1 mg/ml BSA) GF/C filters; the filters were then washed with a total of five 1-ml aliquots of the same buffer. The filters were dried at 80° and the filter-bound radioactivity was determined in a Packard β-counter (34.6% counting efficiency), using 2 ml of CytoScint (ICI Biomedicals, Inc., Irvine, CA) in miniature polyethylene vials. The peptides were analyzed at six to nine concentrations varying over 3 orders of magnitude in three to five separate binding experiments with at least five different synaptosome preparations, to reduce the inherent variability between each preparation.

Total binding was quantitated by determining the ratio of bound to free, or unbound, labeled ligand (*B*/*F* ratio). This accomodates consideration of both the residual radioactivity on the filter (20–60 cpm) and the nonspecifically bound labeled ligand in the presence of excess (2 μM) unlabeled DAGO or DADLE to oversaturate μ or δ binding sites, respectively. The extent of specific binding by [^{3}H]DAGO was 8.7 ± 0.4% (range, 6.9–10.4%; 2200–3300 cpm) and that by [^{3}H]DADLE, 5.0 ± 0.4% (range, 3.4–7.8%; 1000–2300 cpm), which are similar to published data (35).

The IC_{50} values (nM) represent the ligand concentration required to displace 50% of the radioisotope-labeled DAGO (μ sites) or DADLE (δ sites). These values are consistently very close to values published for these peptides (32, 34–36).

Linear regression analysis was carried out using SigmaPlot, version 3.10 (Jandel Scientific, Corte Madera, CA), for the correlation between receptor binding studies with biological activity (8, 9).

Results

Binding to μ receptor sites. Displacement curves for dermorphin and several analogues are illustrated in Fig. 1. These peptides are arbitrarily subdivided according to their relative affinities for the μ receptor, as follows: high affinity, IC_{50} values equal to or less than 1 nM; moderate affinity, IC_{50} values between 2 and 10 nM; and weak affinity, IC_{50} values above 25 nM (Table 1).

Among the high affinity peptides, only [Ser-NHNHZ^7]dermorphin (peptide **23**) bound with greater avidity for μ sites than dermorphin, whereas [Ser(Bzl)^7]dermorphin (peptide **22**) was equipotent (peptide **1**). Other synthetic analogues, [Sar^4]-, [Trp^5]-, [des-Ser^7]-, [Gly^7]-, and [Tyr(Bzl)^{5,7},Hyp^6]dermorphin (peptides **5**, **9**, **14**, **16**, and **21**), and the natural homologue [Hyp^6]dermorphin (peptide **13**), (2) were able to effectively compete with [^{3}H]DAGO at μ sites. In fact, dermorphin (peptide **1**), [des-Ser^7]dermorphin (peptide **16**), and [Hyp^6]dermorphin were the only peptides that exhibited greater μ-selectivity than DAGO (peptide **24**); however, only [des-Ser^7]dermorphin was more μ-selective than dermorphin. The IC_{50} values for [Tyr(Bzl)^{5,7},Hyp^6]dermorphin (Table 1) were similar to published data (15, 16).

The largest number of dermorphin analogues exhibited moderate μ receptor affinity. Reduced μ binding resulted from the following modifications in peptide structure: substitution of D-Ala^2 by D-Met (peptide **2**); removal of the carboxy-terminal amide group (peptide **3**); substitution of an aromatic residue at position 4 (peptides **4** and **6**); addition of a Bzl protective group or the loss of the hydroxyl group, or both, at residues 5 in [Tyr(Bzl)^5,des-Ser^7]dermorphin (peptide **17**) or at both residues 5 and 7 (peptides **20** and **21**); and the replacement of Pro^6 by Gly or Val (peptides **11** and **12**). Although the Phe^5 substitution diminished μ receptor selectivity, the effect was less than that observed by Darlak *et al.* (13).

Dermorphin analogues exhibiting the weakest affinity for μ sites included peptides with the following attributes: blocked hydroxyl or amine function on Tyr^1 (peptides **18** and **19**); deletion of Tyr^5 (peptide **15**), which yields a [Pro^5,Ser-NH_2^6]-dermorphin analogue; and replacement of Gly^4 by Pro or of Tyr^5 by Gly (peptides **7** and **10**, respectively). Of these, [des-Tyr^5]-dermorphin and Boc-dermorphin (peptide **19**) had the least affinity for μ binding sites.

The double-reciprocal plot of the apparent μ and δ affinities (Fig. 2) provides another method to represent receptor site selectivity (36); dermorphin and all the analogues studied interact with μ sites. A plot of the IC_{50} values of the high affinity

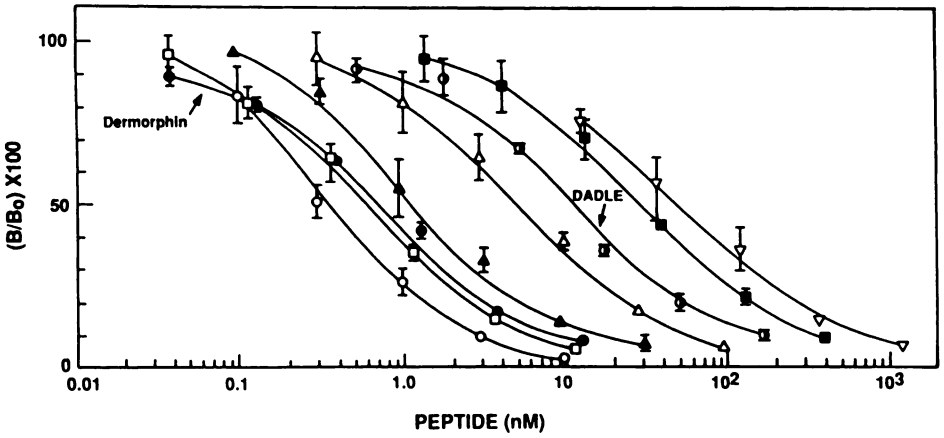

Fig. 1. Displacement of [^{3}H]DAGO by dermorphin and dermorphin analogues in a μ receptor assay. Details are given in Experimental Procedures. ●, dermorphin; ◑, DADLE; ○, [Ser-NHNHZ^7]dermorphin; □, [Ser(Bzl)^7]dermorphin; ■, [Gly^5]dermorphin; ▲, [Tyr(Bzl)^{5,7},Hyp^6]dermorphin; △, [Tyr(Bzl)^{5,7}]dermorphin; ▽, [Pro^4]dermorphin. *Error bars*, standard error for each *point*; their absence indicates that the standard error lies within the dimensions of the *symbol*. Peptide ligand (nM) is plotted against the percentage of the ratio of the binding in the presence of ligand (B) to that in its absence (B_0), derived from the B/F ratio.

TABLE 1

Binding parameters for dermorphin analogues to rat brain synaptosomes

The IC_{50} values were obtained according to the methods described in Experimental Procedures. *Lines*, sequence identity to dermorphin (peptide 1); *open boxes*, an amino acid deletion. Boc-dermorphin (peptide 19) is dermorphin with the α-function protected by the Boc protecting group. Peptides 24 and 25 are the enkephalin analogues DAGO and DADLE, respectively. The μ-selectivity ratio (SR) is the ratio $IC_{50}^{\delta}/IC_{50}^{\mu}$ (36). Values are mean ± standard error.

	Peptide	IC_{50}		SR
		μ	δ	
		nM		
1	Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2	0.68 ± 0.05	161.7 ± 6.06	237.8
2	— D-Met —	8.20 ± 0.15	81.7 ± 5.46	10.0
3	— OH	2.43 ± 0.52	131.7 ± 8.41	54.2
4	— Gly-Phe —	1.78 ± 0.71	10.8 ± 0.64	6.1
5	— Sar —	1.09 ± 0.21	132.3 ± 13.5	121.4
6	— Phe —	2.83 ± 0.46	267.0 ± 101.8	94.3
7	— Pro —	38.7 ± 6.77	1,003.3 ± 126.7	25.9
8	— Phe —	2.88 ± 0.76	80.3 ± 6.49	27.9
9	— Trp —	0.73 ± 0.09	80.3 ± 5.33	110.0
10	— Gly —	27.9 ± 4.03	431.7 ± 57.8	15.5
11	— Gly —	1.71 ± 0.36	70.0 ± 15.3	40.9
12	— Val —	3.27 ± 0.55	17.2 ± 2.04	5.3
13	— Hyp —	1.02 ± 0.08	164.3 ± 6.7	161.1
14	— Gly —	0.93 ± 0.19	102.7 ± 21.2	110.4
15	— □ —	294.3 ± 65.6	1,683.3 ± 16.7	5.7
16	— □ —	0.86 ± 0.13	266.3 ± 17.0	309.7
17	Bzl Tyr — □ —	3.55 ± 0.05	31.0 ± 3.06	8.7
18	Bzl Tyr —	31.3 ± 2.40	2,687.0 ± 557.0	85.8
19	Boc-Tyr —	356.7 ± 44.1	14,525.0 ± 6,174.0	40.7
20	Bzl Bzl Tyr — Ser —	4.88 ± 1.21	24.2 ± 2.68	5.0
21	Bzl Bzl Tyr-Hyp-Ser —	1.03 ± 0.30	16.4 ± 3.96	15.9
22	Bzl Ser —	0.61 ± 0.07	2.97 ± 0.42	4.9
23	— Ser-NHNHZ	0.44 ± 0.02	48.3 ± 4.97	109.8
24	— Gly-*N*-Me-Phe-Gly-ol	1.36 ± 0.37	203.5 ± 18.8	149.6
25	— Gly-Phe-D-Leu-OH	12.2 ± 1.16	0.27 ± 0.03	0.02

dermorphin analogues against their bioactivities in the guinea pig ileum bioassay, adapted from the data of de Castiglione *et al.* (8) and Melchiorri *et al.* (9), is presented in Fig. 3. There was a significant correlation between apparent receptor affinities and effectiveness of the peptides in facilitating relaxation of electrically stimulated smooth muscle preparations, in which μ-type receptors predominate.

Binding to δ receptor sites. Dermorphin and its analogues generally exhibited little ability to displace [^{3}H]DADLE from δ binding sites; curves for displacement by dermorphin and several analogues relative to DADLE are shown in Fig. 4. The dermorphin competition curves were parallel to that of DADLE (only those peptides whose IC_{50} values differed by a factor of 2 or more from each other are represented in the figure, for the sake of clarity). In the absence of [*N*-Me-Phe^3,D-Pro^4]morphiceptin, biphasic nonparallel displacement curves of labeled DADLE frequently occurred.

Numerous dermorphin analogues (peptides **2**, **4**, **8**, **9**, **11**,

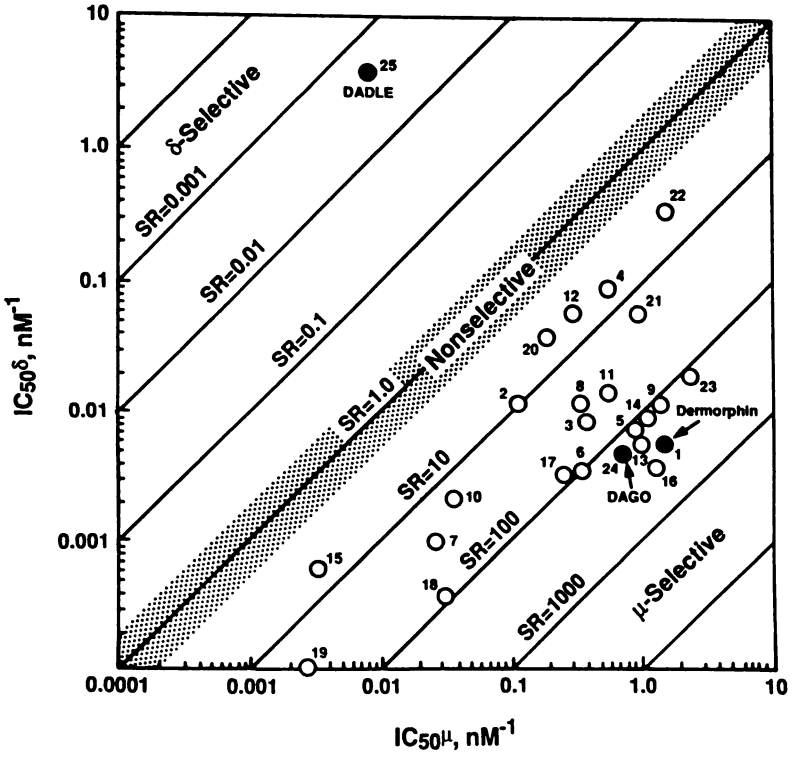

Fig. 2. Double-reciprocal plot of the IC_{50} values indicates the relationship between dermorphin, dermorphin analogues, DAGO, and DADLE. The *numbered symbols* refer to peptides listed in Table 1. SR is the selectivity ratio, defined in Table 1. The region of nonselectivity is *stippled* (36).

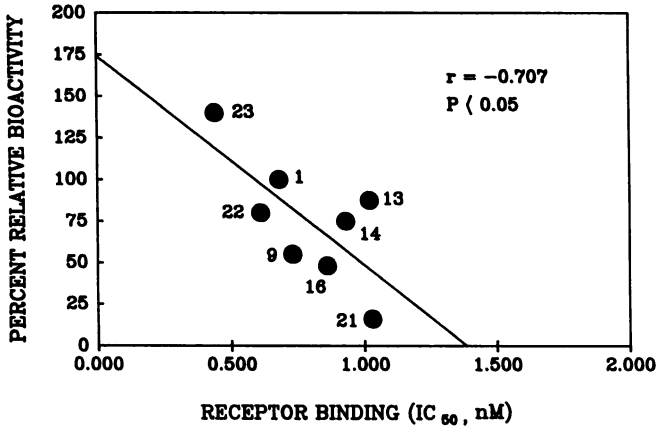

Fig. 3. Association between binding to high affinity μ receptors in rat brain synaptosomes and the relaxation of electrically stimulated guinea pig ileum. Regression analysis ($r = -0.707$, $p < 0.05$) of the percentage of relative bioactivity of dermorphin analogues (Table 1), compared with dermorphin (arbitrarily set at 100%) (8, 9), versus receptor binding (IC_{50}) is shown.

12, 17, and 20–23) exhibited apparent affinities for δ subsites 2- to 54-fold higher than that of dermorphin; however, none approached that of DADLE (peptide 25) (Table 1). Only two peptides, [Gly^2,Phe^4]- and [Ser(Bzl)^7]dermorphin (peptides 4 and 22) (Table 1), had IC_{50} values of approximately 10 nM or less; the sequence of the former peptide is similar to that of the enkephalins (for comparison, see DADLE, peptide 25). Examination of the selectivity ratios indicated that some dermorphin analogues (peptides 4, 12, 15, 17, 20, and 22) exhibit a significant shift toward δ subsite preference, although they all remained μ-selective (Fig. 2). In comparison, DADLE exhibited selectivity for δ-type receptors, with a μ-selectivity ratio of 0.02 (Table 1). Furthermore, no correlation was observed between dermorphin δ receptor affinities in brain synaptosomes and bioactivity data from mouse vas deferens preparations (8, 9) (not shown).

Discussion

Data presented in this communication support the hypothesis that μ-type opioid receptor selectivities and affinities for peptides related to dermorphin are influenced by both solution conformation(s) of the ligands (22, 25, 29, 30) and significant molecular association (hydrophobic or electron donor-acceptor interactions, or both) interactions involving aromatic groups of the peptide and complementary receptor sites. Structure-binding relationships support the idea that orientation of the backbone of dermorphin permits optimal interaction with the μ receptor when amino acid residues 1, 3, and 5 contain aromatic groups (25, 27, 29, 30, 37).

One hydrophobic P$^\mu$ site in the μ receptor is assumed to complement the dermorphin Phe^3 residue (Fig. 5). Our results support published data (37) that the chemical and spatial characteristics of the Phe^3 residue are important in orienting the peptide in the receptor (29, 30). However, the P$^\mu$ site may differ from the "P site" suggested in the binding of Phe^4 of enkephalins and endorphins to δ receptors (38). Modification of the aromatic ring of Phe^3 in dermorphin *N*-terminal tetrapeptides (37) or its substitution in dermorphin (peptide 4) brought about diminished biological activity (37) and μ binding properties (Table 1).

A "T site" represents a second region within the opioid receptor that interacts with the Tyr^1 phenolic group of enkephalin (22, 38). In the case of dermorphin, a T$^\mu$ site may interact with and accommodate both Tyr^1 and Tyr^5 (Fig. 5), due to the stacking between the phenolic rings of these two residues (26). Dermorphin was assumed to acquire a folded structure upon interaction with a hydrophobic membrane pocket (30). This conclusion was based on both NMR (21, 22, 30) and CD (27)

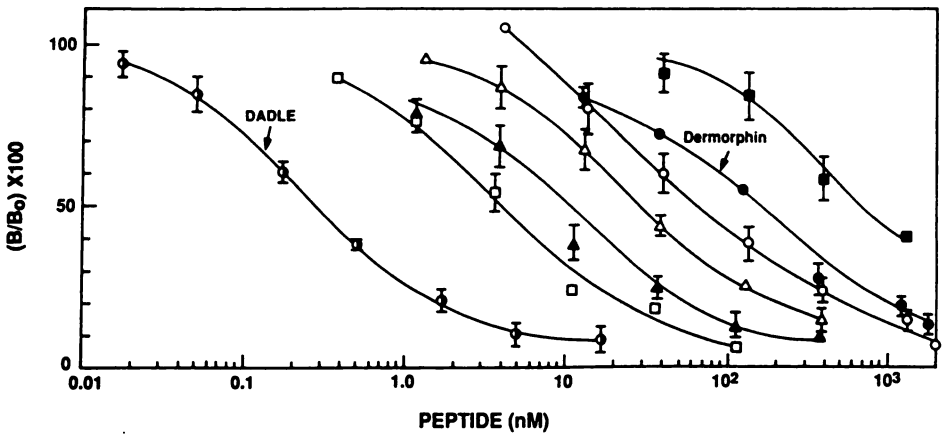

Fig. 4. Competition curves for dermorphin, DADLE, and dermorphin analogues in a δ receptor binding assay. The peptides, symbols, and coordinates are those listed in the legend to Fig. 1, except that [Pro⁴]-dermorphin is omitted because its displacement curve would lie outside the figure limits (i.e., >1 μM).

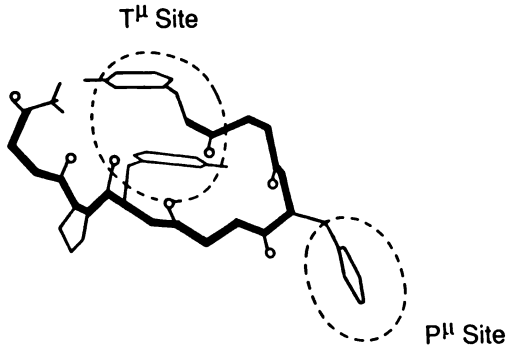

Fig. 5. Schematic representation of the solution conformation of dermorphin and μ receptor sites that complement the aromatic groups at positions 1, 3 and 5. The *heavy solid line* indicates the peptide backbone, modified from Pattabiraman *et al.* (26) who based their representation on NMR studies, which indicate intramolecular hydrogen bonding of a folded dermorphin structure (25–27, 30). The Pᵘ site at Phe³ and a Tᵘ site (involving the stacked phenolic groups of Tyr¹ and Tyr⁵) are represented as *dashed lines* in the μ opioid receptor. The phenyl group of Phe³ extends outward from the folded peptide (26) and was proposed to fit into a hydrophobic pocket with the shape of an oblate ellipsoid (29). The Tᵘ site may differ from the "T site" (38) in that it has the ability to participate in hydrogen bonding and electron donor-acceptor interactions with either or both of the phenolic groups of Tyr¹ and Tyr⁵ in the folded dermorphin molecule (26, 29). Further, the presence of Bzl or NHNHZ groups of Ser⁷ (peptides **22** and **23**) may stabilize the folded solution conformation of dermorphin and assist in anchoring the peptide to the receptor through hydrophobic interactions.

studies of dermorphin analogues complexed to crown-ethers, which are used to simulate membrane environmental characteristics (27, 28, 30, 39).

NMR studies have indicated that several characteristics of a Tᵘ site are influenced by solution properties of dermorphin and various analogues (21–26, 28–30): (a) the phenyl groups of Tyr¹ and Tyr⁵ freely rotate around the β,γ carbon bond (24), (b) the *N*-terminal sequence forms a type II′ β-turn (28, 30), and (c) the peptide assumes a folded structure (21, 23, 26, 28). Therefore, it is plausible that the closely stacked aromatic regions of Tyr¹ and Tyr⁵ participate in molecular association with the μ binding site (Fig. 5). Furthermore, the integrity of the internal tripeptide sequence Phe³-Gly⁴-Tyr⁵ appears to provide a significant contribution to the solution conformation of dermorphin, due to intramolecular hydrogen bonds (24, 26) involving (a) Tyr¹ NH and Gly⁴ C═O, (b) Tyr¹ C═O and Gly⁴ NH, and (c) Tyr¹ NH and Tyr⁵ C═O (26). This region is also involved in the formation of the β-turn (26, 29, 30), which presumably leads to the appropriate alignment of dermorphin within the μ receptor. It should be noted that, although the *N*-terminal tetrapeptide amide represents the minimal bioactive structure (7, 8, 15, 21, 40, 41), it only weakly interacts with membrane receptor sites (15, 18).

Support for the importance of an energetically favored solution conformation in determining apparent affinities of dermorphin analogues for μ receptor sites is provided by our results (Figs. 1 and 4, and Table 1) and by other studies using Phe³-modified analogues (27, 29, 30, 37). For instance, [des-Tyr⁵]-dermorphin (peptide **15**) and [Gly⁵]dermorphin (peptide **10**) have markedly lower affinities for μ and δ sites, whereas [Phe⁵]-dermorphin (peptide **8**) and [Trp⁵]dermorphin (peptide **9**) exhibit apparent affinities reasonably close to that of dermorphin; the differences in affinities may be attributable to spatial orientation, charge distribution in the associated aromatic residues, or both. NMR studies indicate that the aromatic ring of Phe³ of dermorphin appears to assume a low energy conformation (28) resulting from peptide folding; this enables the Phe³ phenyl ring to extend outward from the peptide backbone (26) in a *cis* arrangement (i.e., in the same plane) with Tyr¹ (29, 37), thereby enhancing binding to μ receptors (42).

The significance of the proper alignment of the dermorphin backbone and the side chains of its constituent residues relative to attachment sites in the μ receptor site is exemplified by the diminished affinity observed for [Pro⁴]dermorphin (peptide **7**). Proline disrupts the solution conformation of dermorphin by misaligning Tyr¹ relative to Tyr⁵, thereby interfering with the stacking of their phenolic residues. The resulting spatial orientation of Phe³ relative to the peptide backbone and the tyrosine residues is additionally altered because Pro⁴ would decrease the shielding of D-Ala² by either Tyr¹ or Phe³ (24); this modifies the diamagnetic shift in the resonance between the D-Ala² methyl group and the aromatic ring of Phe³ (25, 29) and alters the shielding of Gly⁴ NH (24), which hydrogen bonds with the Tyr¹ C═O (26, 40). NMR studies on dehydro-Phe-dermorphin analogues (30) and CD data (27) indicated that the intramolecular hydrogen bond between Tyr¹ C═O and Gly⁴ NH depends on Phe³. The presence of proline at position 5 in [des-Tyr⁵]dermorphin (peptide **15**) obviates the stacking of Tyr¹ and Tyr⁵, changes the bond angle in the peptide backbone, and alters interactions involving the C-terminal residues (23, 26). These proline substitution analogues provide evidence that both the N- and C-terminal sequences of dermorphin are in-

volved in optimal interaction with the μ receptor and can be correlated with biological activities (Fig. 3) (8, 9).

The presence of a Bzyl protecting group in selected dermorphin analogues (peptides **17** and **20–22**) generally enhanced the displacement of the radiolabeled ligand from δ subsites by 7- to 54-fold, relative to those peptides lacking these groups (peptides **1**, **13**, and **16**). Interestingly, the affinity of [Ser(Bzl)^7]dermorphin (peptide **22**) or [Ser-NHNHZ^7]dermorphin (peptide **23**) for μ sites equals or surpasses that of dermorphin. The presence of these Bzl groups on dermorphin influences multiple receptor site selectivity through hydrophobic interactions, by enhancing the association between the phenolic groups of the tyrosine residues, which could stabilize a C-terminal β-turn (26, 30).

The concept of "membrane-assisted opioid receptor selection" (43, 44) postulates that (a) an "anionic fixed-charge compartment" enables peptides with a net positive charge to preferentially interact with the μ subsite (19), (b) μ receptor affinity is adversely affected by hydrophobic substituents on the peptide (44), and (c) δ subsites are proposed to possess a positively charged recognition site located on the receptor protein (44). Our results (Table 1), however, confirm that the affinities and selectivities of dermorphin and structural analogues for rat brain μ-type opioid receptors are significantly influenced by specific hydrophobic residues and by the solution conformation of the peptides. The intramolecular hydrogen bonds of a folded dermorphin molecule were shown to permit optimal association between the tyrosine residues (24, 26, 30); one consequence is that the Phe^3 phenyl residue is free to interact with a complementary component of the receptor, i.e., the membrane μ receptor contains hydrophobic regions or pockets that facilitate peptide binding through nonionic mechanisms.

The importance of receptor conformation was also suggested from NMR studies with the deltorphins (5). These are peptides that exhibit exceptionally high affinity and selectivity for only δ receptor binding sites (3–6, 10, 11) and yet contain the generalized tripeptide sequence of Tyr^1-D-Xxx^2-Phe^3 found in dermorphin. The methyl group of D-Ala^2, located between the aromatic rings of Tyr^1 and Phe^3, imposes restrictions on the flexibility of the peptide backbone (25, 30). Considering the presence of this common sequence in both μ- and δ-selective ligands [e.g., the dermorphins and deltorphins (6)], it would appear that discrimination between receptor subsites depends on specific residues in the C-terminal region of the peptide (6, 10, 21, 25). Our data also suggest that selectivity for receptor recognition involves residues capable of participation in both hydrophobic (21, 25, 29, 30) and electrostatic interactions with the receptor (19, 38, 43, 44).

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