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Opioid receptor selectivity alteration by single residue replacement: synthesis and activity profile of [Dmt^1]deltorphin B

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Abstract

The single amino acid replacement of 2′,6′-dimethyl-L-tyrosine in deltorphin B (H-Dmt-D-Ala-Phe-Glu-Val-Val-Gly-NH_2) yielded high affinity for μ- and δ-binding sites. [Dmt^1]Deltorphin B lacks activity at κ-opioid binding sites. Bioactivity in vitro with guinea-pig ileum confirmed that [Dmt^1]deltorphin B interacted with μ-opioid receptors by reducing electrically induced contractions in a naloxone-reversible manner and was 150-fold more potent than morphine and comparable to [D-Ala^2,NMePhe^4,Gly-ol^5]enkephalin (DAGO). The inhibition of spontaneous contractions of rabbit jejunum provided evidence for δ-opioid receptor interaction. Analgesia (hot plate and tail flick tests) revealed that [Dmt^1]deltorphin B was 180- to 200-fold more potent than morphine. Pretreatment with naloxone, naltrindole or H-Dmt-Tic-Ala-OH (a highly selective δ-opioid receptor antagonist) prevented [Dmt^1]deltorphin B antinociception. Thus, [Dmt^1]deltorphin B exhibited remarkably high dual affinity and bioactivity toward δ- and μ-opioid receptors.

Keywords: Antinociception; Bioassay; Deltorphin; Opioid receptor; Peptide synthesis

1. Introduction

The existence of at least three different classes of opioid receptors (μ-, δ- and κ-opioid receptors) is well established (Martin et al., 1976; Robson et al., 1983). While endogenous ligands (enkephalin, β-endorphin and dynorphin) show generally poor selectivity for opioid receptors, many synthetic peptide analogues of the mammalian peptides exhibit enhanced selectivity for one or another of the receptors and their pharmacologically defined subtypes (Hruby and Gehring, 1989), such as δ_1- and δ_2-opioid receptor subtypes (Qian et al., 1994). The recent finding that intracerebroventricular administration of an antisense oligodeoxynucleotide complementary to the cloned rat δ receptor blocks deltorphin antinociception (δ_2-opioid receptor-mediated response), and not [D-Pen^2,D-Pen^5]enkephalin antinociception (δ_1-opioid receptor-mediated response), provides evidence for the existance of δ-opioid receptor subtypes (Lai et al., 1994).

Current evidence indicates that analgesia mediated by agonist activation of δ-opioid receptors (Heyman et al., 1988) produces less marked side-effects than analgesia mediated by μ-opioid receptors (Galligan et al., 1984). The classic symptoms associated with alkaloid opiates such as morphine, which interacts with μ-opioid receptors, include respiratory and gastrointestinal depression, and the development of tolerance and addiction. The absence of a naturally occurring opiate (non-peptide opioid) endowed with high δ-opioid receptor selectivity had generally discouraged investigations into the role of the δ-opioid receptor in modulating analgesia and other pharmacological activities to counter morphine dependence. However, newer synthetic preparations of oxymorphindole and related derivatives (Portoghese et al., 1988), and (+)-4-[(αR)-α-((2S,5R)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxy-

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benzyl]-*N*,*N*-diethylbenzamide (Calderon et al., 1994) now provide potential non-peptide ligands for studies on δ-opioid receptors. Nonetheless, clinical relevance for these drugs has yet to be thoroughly assessed.

Deltorphins, on the other hand, which represent the most δ-opioid receptor-selective and potent family of opioid peptides originally isolated from frog skin (Erspamer et al., 1989), have served as the parent peptide in the synthesis of numerous active opioid peptide analogues. This stimulated a new investigation of the structural characteristics of ligands that interact with δ-opioid receptors. Studies on 2′,6′-dimethyl-L-tyrosine (Dmt)-substituted analogues centered on the mediation of analgesia on opioid receptors (Hansen et al., 1992). The presence of Dmt in the selective δ-opioid di- and tripeptide receptor antagonists (Temussi et al., 1994; Salvadori et al., 1995) suggested that Dmt confers a conformation to the peptide similar to that of its bioactive conformer (Bryant et al., 1996).

Our investigations were further undertaken to modify deltorphin B (Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH_2) subtly by replacing Tyr with Dmt and to determine the activity of [Dmt^1]deltorphin B in pharmacological bioassays and radioligand binding assays for μ- and δ-opioid receptors. Pharmacological bioassay was performed with both in vivo and in vitro experimental procedures.

Recently, it has been proposed that hot plate analgesia in mice is mainly due to the activation of supraspinal μ-opioid receptors whereas tail flick analgesia at the spinal levels appears to involve mainly δ-opioid receptor activation (Porreca et al., 1984). Therefore, in our study, in order to establish whether [Dmt^1]deltorphin B induces analgesia through μ- and /or δ-opioid receptor activation, we studied analgesia with both the hot plate and the tail flick test. To establish clearly that [Dmt^1]deltorphin B induces analgesia through μ- or δ-, or both receptor types its opioid receptor binding characteristics in rat brain membranes were compared to its activity in various pharmacological tests in vitro and in vivo.

2. Materials and methods

2.1. Peptide synthesis

[Dmt^1]Deltorphin B was prepared according to the solid phase methods as reported for the synthesis of deltorphin B and deltorphin C (H-Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2) (Bryant et al., 1994) starting with Fmoc-PAL-PEG-PS-resin (0.19 mmol/g, 0.11 mmol). Amino acids were Fmoc-protected and *tert*-butyl ester (*O*-*tert*-butyl) was used to mask the side chain of Glu. Amino acids were coupled for 1 h in a 4-fold excess of diisopropylcarbodiimide in the presence of a 4-fold excess of 1-hydroxybenzotriazole; double coupling was required in the Val-Val and Glu-Val acylation step. Piperidine/*N*,*N*-dimethylformamide (20%, v/v) was employed for removal of the N^{α}-Fmoc group following each coupling step; coupling reactions were monitored by the Fmoc-piperidine adduct at 365 nm.

2.2. Peptide purification

Crude Boc-Dmt-D-Ala-Phe-Glu(*O*-*tert*-butyl)-Val-Val-Gly-PAL-PEG-PS-resin (0.67 g) was treated at room temperature for 1 h in a solution containing 88% trifluoroacetic acid, 7% triethylsilane, 5% water (15 ml/g). The crude material (85% purity) was further purified by preparative high-performance liquid chromatography (HPLC) using a Waters Delta Prep 3000 system with a Delta Pak C_{18} 300 A (30 × 300 mm, 15 μm, spherical) column. Elution was conducted using a linear gradient from 30% to 60% acetonitrile containing 0.1% trifluoroacetic acid over 30 min at a flow rate of 30 ml/min. The purity of the product was assessed by analytical HPLC using a Bruker LC-21 instrument fitted with Bruker LC 313 UV variable wavelength detector and a Vydac C_{18} (150 × 4.6 mm 5 μm particle column) with mobile phases A and B composed of 10% acetonitrile in 0.1% trifluoroacetic acid and 60% acetonitrile in 0.1% trifluoroacetic acid, respectively. Capacity factor (K') of the peptide was determined with a linear gradient from 0% B to 100% B at a flow rate of 1 ml/min over a 25-min time interval and were monitored at 220 nm.

Thin layer chromatography was done with precoated silica gel F_{254} plates (Merck, Darmstadt, Germany) in the following solvent systems: **I**, 1-butanol/acetic acid/H_2O (3:1:1, v/v/v) and **II**, ethyl acetate/pyridine/acetic acid/H_2O (12:4:1.2:2.2, v/v/v/v). Amino acid analysis was carried out using a phenylisothiocyanate as the amino acid derivatization reagent. The yield of peptide **1** was 57% with a purity estimated at > 99%. Analytical data: TLC R_f **(I)** 0.61, **(II)** 0.34; K' 5.74; MS m/z 811 (MH^+). Amino acid analysis: Dmt 0.92, Ala 1.01, Phe 0.98, Glu 0.97, Val 1.86, Gly 1.0.

2.3. Receptor binding assays

Rat brain membrane synaptosomes (P_2 fraction) were prepared as described previously (Lazarus et al., 1989) and stored at −80°C in buffered glycerol containing protease inhibitors. The δ- and μ-opioid receptor binding sites were specifically labelled using [^{3}H][D-Pen^{2,5}]enkephalin (DPDPE) and [^{3}H]DAGO, respectively (Bryant et al., 1994). Mixtures from competitive displacement assays were incubated for 2 h at room temperature (22–23°C), rapidly filtered onto glass fiber filters and the membranes were washed repeatedly with ice-cold 0.05 M Tris-HCl containing 0.1% bovine serum albumin. The affinity constants (K_i) according to Cheng and Prusoff (1972) were derived from the competitive binding curves. In order to obtain statistically sufficient dosage points for the determination of the Hill coefficients (η), 25-35 peptide concen-

trations were assayed in triplicate δ- and μ-opioid receptor assays in 3–5 separate binding experiments.

2.4. Data analysis

Receptor binding to δ- and μ-opioid receptor types was determined with the highly stringent iterative calculations published by Attila et al. (1993) and Bryant et al. (1994) in which fits for the two-site binding model are considered valid only when the Hill coefficient (η) is < 0.85 with a narrow log of the 95% confidence intervals and $P < 0.0001$ in the F-test between one- and two-site models. Analyses were conducted using the equations contained within InPlot (v. 4.0) (MS-DOS version) and Prism (v. 1.03) (Windows version) (GraphPad Software, San Diego, CA, USA).

2.5. Antinociception

To determine the nociceptive threshold of the mice, the hot plate test as described previously (Capasso et al., 1993) and the tail flick test according to Capasso et al. (1992) were used.

Drugs used were dissolved in sterile distilled water for intracerebroventricular administration immediately prior to injection at 5 μl/kg or 5 μl mouse, respectively. The i.c.v. injection was performed according to Haley and McCormick (1957) and the site of administration of the peptide was verified in all animals by the injection of 1% methylene blue and examination of the dye distribution in the cerebral ventricles at the termination of the experiment. To evaluate the hot plate and tail flick test responses detailed below, a control latency (T_0) was obtained from the mean of two latencies determined prior to drug injection; test latencies (T_1) were determined at various times after injection for each animal. The percentage of analgesia was calculated as $(T_1 - T_0)/(T_2 - T_0) \times 100$, where the cut-off times (T_2) for the hot plate and tail flick tests were 60 and 15 s, respectively. The median antinociceptive dose (ED_{50}) and 95% confidence limits were calculated according to the method of Litchfield and Wilcox (Tallarida and Murray, 1987).

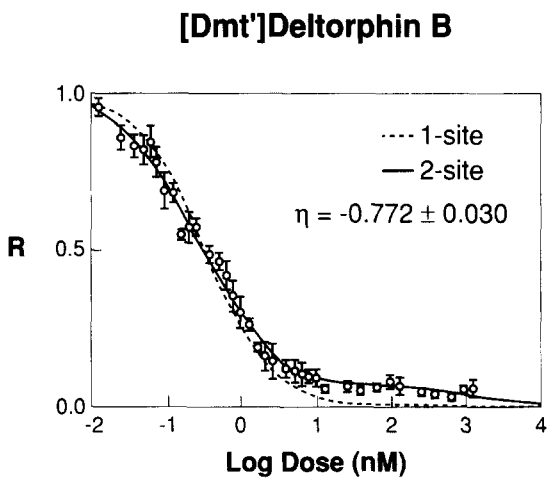

Fig. 1. Goodness of fit of [Dmt^1]deltorphin B to one- and two-site binding models for the δ-opioid receptor. The Hill coefficient (η) was determined according to Attila et al. (1993) and Bryant et al. (1994) as detailed in section 2.4. The dashed line represents a one-site binding curve and the solid line is the fit to a two-site binding model. The open circles represent the means ± S.E. ($n = 7$); the absence of bars indicates that the S.E. lies within the circle designated as the mean value. R is the ratio of binding in the presence and absence of ligand.

3. Results

3.1. Receptor binding affinity

The replacement of Tyr^1 in deltorphin B by Dmt resulted in a large (630-fold) increase in μ-opioid affinity, while the affinity for the δ-opioid receptor remained unchanged (Table 1). The compounds listed in Table 1 lack affinity at the κ-opioid receptor site; i.e., interaction only occurred at elevated μM concentrations. [Dmt^1]Deltorphin B (**1**) exhibited a weak (8-fold) selectivity toward δ-opioid receptors; the degree of selectivity was 690-fold less than that of the parent compound, deltorphin B (**2**), and 150-fold less than that of deltorphin C (**3**).

Table 1
In vitro characterization of [Dmt^1]deltorphin B and related peptides

Compound	K_i, nM		$K_i\,\mu/K_i\,\delta$	ED_{50}, nM	
	μ	δ		Guinea-pig ileum	Rabbit jejunum
(**1**) [Dmt^1]Deltorphin B	1.01 ± 0.06 (3)	0.13 ± 0.027 (3)	7.7	300 (216–353)	360 (297–395) [a]
(**2**) Deltorphin B	638 ± 55 (5)	0.12 ± 0.03 (6)	5317	–	850 (775–915)
(**3**) Deltorphin C	272 ± 50 (11)	0.24 ± 0.06 (6)	1135	–	730 (665–794)
Morphine	–	–		43 μM (38–52)	–
DAGO	1.51 ± 0.18 (8)	376.3 ± 81.8 (4)	0.004 [b]	22.0 (18.3–24.9)	–

The affinity constants (K_i) are means ± S.E. with n values in parentheses. Selectivity for the δ-opioid receptor is the ratio of the K_i values (μ/δ). The data in the guinea-pig ileum bioassay are given as the ED_{50} with 95% confidence limits in parentheses. [a] $P < 0.01$ vs. **2** and **3**. [b] Taken from Lazarus et al. (1989).

Table 2
In vivo antinociception activity of [Dmt^1]deltorphin B and related peptides

Compound	Hot plate test		Tail flick test	
	i.c.v., ED_{50} nM	Rel. pot.	i.c.v., ED_{50} nM	Rel. pot.
(1) [Dmt^1]Deltorphin B	0.016 (0.011–0.030) [a]	180	0.011 (0.0087–0.023)	300
(2) Deltorphin B	0.021 (0.016–0.042)	140	0.0038 (0.0016–0.008) [b]	840
(3) Deltorphin C	0.035 (0.020–0.051)	80	0.017 (0.01–0.021)	190
Morphine	2.88 (1.75–3.9)	1	3.18 (2.9–3.7)	1

The data are given as ED_{50} with 95% confidence limits in parentheses. [a] $P < 0.01$ vs. **3**; [b] $P < 0.01$ vs. **1** and **3**.

Detailed statistical analyses of η for the δ-opioid receptor site for peptide **1** demonstrated a best fit for a heterogeneous two-site binding model (η, -0.772 ± 0.030; log 95% confidence interval, -0.524 to -0.442; r^2, 0.987633; F 21.9; DF, 2.34; $P < 0.0001$) (Fig. 1), whereas the high affinity binding to μ-opioid receptors fitted a single, bimolecular one-site binding model ($\eta = -1.097 \pm 0.025$). Deltorphin C (**3**) also fitted a one-site binding model for both δ- and μ-opioid receptors (Bryant et al., 1994).

3.2. Bioactivity

The high μ-opioid receptor affinity of **1** was confirmed in the guinea-pig ileum bioassay in vitro run as described previously (Persico et al., 1991). These experiments indicated that peptide **1** reduced the electrically induced contractions of guinea-pig ileum while peptides **2** and **3** failed to cause significant inhibition up to μM concentrations (Table 1). Since naloxone at a concentration of 0.05 nM reversed the inhibition effect of **1**, the results are entirely consistent with an interaction of μ-opioid receptors. Furthermore, in a comparative study performed with morphine (a preferential μ-opiate receptor alkaloid ligand) and DAGO (a highly μ-selective opioid peptide), peptide **1** appears to be 150-fold more active than morphine (Table 1) and as active as DAGO. The strong inhibition of the spontaneous contractions of rabbit jejunum by peptide **1**, evaluated following the protocol of Valeri et al. (1992), also indicated an interaction with δ-opioid receptors since this effect was totally blocked by naltrindole, an established δ-opioid receptor antagonist, at an equimolecular concentration. Moreover, in comparison to peptides **2** and **3**, compound **1** appeared to be moderately more active as an inhibitor than either of the deltorphins, which were comparable to each other as to activity (Table 1).

3.3. Antinociception

The results obtained with the hot plate test indicated that 10 min after i.c.v. administration, peptide **1** induced a significant and dose-related analgesia in the mouse (Table 2), which lasted for the entire experimental period (45 min). In a companion study with morphine, peptide **1** was 180-fold more potent than this narcotic opiate alkaloid, thus confirming the observations on the interaction with μ-opioid receptors with the in vitro bioassays and receptor binding (Table 1).

These observations were further confirmed with the tail flick test, in which peptide **1** had an analgesic activity comparable to that demonstrated in the hot plate test. Whereas peptide **2** was about 3 times more active than peptide **1**, all three deltorphin analogues (**1–3**) were about 300- to over 800-fold more analgesically potent than morphine (Table 2). A concurrent study then considered the effects of naltrindole (δ-opioid receptor antagonist), naloxone (μ-opioid receptor antagonist), and H-Dmt-Tic-Ala-OH (DTA), a new highly selective δ-opioid receptor antagonist (Salvadori et al., 1995) on the analgesic effect induced by peptides **1** and **3** (Table 3). While DTA and naltrindole (0.05 nM, i.c.v.) produced no measurable antinociception, both antagonized the antinociceptive action during co-administration of peptides **1** or **3** in the tail flick test (Table 3). Identical treatment with reported δ receptor antagonists failed to affect antinociception in the hot plate test (Table 3). On the other hand, pretreatment

Table 3
Co-administration of, or pretreatment with, H-Dmt-Tic-Ala-OH (DTA), naltrindole (NTI) or naloxone (NAL) on the ED_{50} for antinociception produced by i.c.v. [Dmt^1]deltorphin B and deltorphin C in the hot plate test and tail flick test

Peptide	Hot plate test, ED_{50}, nM			Tail flick test, ED_{50}, nM		
	+ DTA	+ NTI	+ NAL	+ DTA	+ NTI	+ NAL
(1) [Dmt^1]Deltorphin B	0.097 (0.065–0.11)	0.067 (0.042–0.081)	1.76 (1.23–1.97)	2.7 (2.9–3.1)	1.9 (1.3–2.1)	1.58 (1.1–1.84)
(3) Deltorphin C	0.053 (0.031–0.083)	0.042 (0.016–0.072)	0.055 (0.01–0.073)	2.3 (1.9–2.9)	1.8 (1.1–2.3)	0.77 (0.65–0.83)

All antagonists were used at a concentration of 0.05 nM. Co-administration (DTA, 0 h) or pretreatment consisted of administration of the antagonist (NTI, 8 h; NAL, 0.5 h) before the peptide. The data are given as ED_{50} with 95% confidence limits in parentheses.

with naloxone (0.05 nM, i.c.v.), which does not produce antinociception, antagonized the activity of peptide **1** in both the hot plate and tail flick tests (Table 3), but it antagonized peptide **3** in the tail flick test and not in the hot plate test (Table 3). Statistical analysis comparing data of Tables 2 and 3 showed significant ($P < 0.01$) differences between peptides tested alone and the same in the presence of each antagonists except for deltorphin C in the hot plate test versus deltorphin C plus each antagonist in the hot plate test.

4. Discussion

The data are the first clear demonstration that a peptide subtly modified in the side-chain of the N-terminus residue yielded an opioid with high dual affinities to both δ- and μ-opioid receptor types. Previously, this phenomenon of opioid infidelity (Lazarus et al., 1996), in which high δ-opioid receptor affinity was preserved while high μ-opioid receptor affinity was acquired had been observed with peptides in which the anionic amino acid in the fourth position was replaced by an amino acid with an aliphatic side-chain (Bryant et al., 1994) or an α,α-dialkyl cyclic amino acid (Breveglieri et al., 1996). Although Dmt could possibly influence the conformation in the N-terminal portion of deltorphin B by reducing the rotation of the Tyr residue (Bryant et al., 1996), this potential alteration had no effect on the iterative fits of the analysis to a two-site binding model since the η for peptides **1** and **2** remained essentially identical. It therefore appears that only the amino acid residue in the fourth position has the ability to modify peptide conformation significantly, leading to the uncovering of δ-opioid receptor subsites (Bryant et al., 1994; Breveglieri et al., 1996). Yet the apparently enhanced hydrophobicity of the Tyr residue and potential changes in peptide topography considerably influence the interaction with the μ-opioid receptor ligand-binding domain.

The in vitro bioassay data (Table 1) confirm the evidence from opioid receptor binding assays that peptide **1** is a unique opioid agonist with potent activities toward both μ- and δ-opioid receptors. Interestingly, in contrast to our data for peptide **1**, a trimethylated analogue of Tyr^1 in deltorphin C yielded a highly active δ-opioid receptor-selective agonist (Qian et al., 1994), whereas the Dmt-D-Ala-arylalkylamides were moderately μ-opioid receptor-selective (Hammond et al., 1994). Thus, the degree of methylation on Tyr^1 affects the conformation of the peptide and its ability to interact differentially with δ- and μ-opioid receptors.

Even though hot plate analgesia is mainly mediated by μ-opioid receptors, it is interesting that the antinociceptive activity of **1** is comparable to that of peptides **2** and **3**. However, considering that the μ-opioid receptor affinities of peptides **2** and **3** are significantly much lower than that of peptide **1**, the data further suggest that δ-opioid receptors appear to be involved, in part, in hot plate analgesia (Porreca et al., 1984). The binding data reported in Table 1, on the other hand, suggest that peptides **1** and **3** behave differentially toward δ- and μ-opioid receptors and this disparate behaviour is also supported by antinociception tests with and without selective opioid receptor antagonists (Tables 2 and 3).

4.1. Conclusions

At present, it appears that the replacement of the Tyr^1 residue by the unnatural Dmt analogue in the δ-selective opioid agonist, deltorphin B, produced a peptide with high affinity for both μ- and δ-opioid receptors, thereby yielding a δ-opioid receptor ligand which, to all intents and purposes, lacks δ-opioid receptor selectivity. While the high affinity for the μ-opioid receptor was confirmed in the in vitro pharmacological assay (guinea-pig ileum), in vivo antinociception apparently failed to correlate with high μ-opioid receptor activity; however, the activity profile of peptide **1** was more closely related to that of the δ-selective opioid peptides **2** and **3**. Similarly, the analgesic activity of the δ-opioid-selective enkephalin analogue [D-Ser(*O-tert*-butyl)^2,Leu^5]enkephalyl-Thr(*O-tert*-butyl)^6 (BUBU) (Baamonde et al., 1991) and [Dmt^1]-DPDPE (Hansen et al., 1992) is mediated through different receptor types: the μ-opioid receptor in the case of BUBU and an unknown δ-opioid receptor subtype for the latter peptide. In summary, our results indicate that [Dmt^1]-deltorphin B possesses remarkable agonist activity and exerts effects on both μ- and δ-opioid receptors in vitro and in vivo, and also that the newly discovered opioid tripeptide antagonist, DTA, exerts antagonistic activity primarily toward the δ-opioid receptor, in keeping with in vitro data.

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