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A comprehensive study on the putative δ-opioid receptor (sub)types using the highly selective δ-antagonist, Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH

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ABSTRACT

The goal of our work was a throughout characterization of the pharmacology of the TIPP-analog, Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH and see if putative δ-opioid receptor subtypes can be distinguished. Analgesic latencies were assessed in mouse tail-flick assays after *intrathecal* administration. *In vitro* receptor autoradiography, binding and ligand-stimulated [^{35}S]GTPγS functional assays were performed in the presence of putative δ_1-(DPDPE: agonist, BNTX: antagonist), δ_2-(agonist: deltorphin II, Ile^{5,6}-deltorphin II, antagonist: naltriben) and μ-(DAMGO: agonist) opioid ligands. The examined antagonist inhibited the effect of DPDPE by 60%, but did not antagonize δ_2- and μ-agonist induced analgesia. The radiolabeled form identified binding sites with K_D = 0.18 nM and receptor densities of 102.7 fmol/mg protein in mouse brain membranes. The binding site distribution of the [^{3}H]Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH agreed well with that of [^{3}H]Ile^{5,6}-deltorphin II as revealed by receptor autoradiography. Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH displayed 2.49 ± 0.06 and 0.30 ± 0.01 nM potency against DPDPE and deltorphin II in the [^{35}S]GTPγS functional assay, respectively. The rank order of potency of putative δ_1- and δ_2-antagonists against DPDPE and deltorphin was similar in brain and CHO cells expressing human δ-opioid receptors. Deletion of the DOR-1 gene resulted in no residual binding of the radioligand and no significant DPDPE effect on G-protein activation. Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH is a highly potent and δ-opioid specific antagonist both *in vivo* and *in vitro*. However, the putative δ_1- and δ_2-opioid receptors could not be unequivocally distinguished *in vitro*.

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1. Introduction

The classification of the δ-opioid receptor subtypes has been based on data from analgesic assays in mice, which showed that the effect of DPDPE (Tyr-c[D-Pen-Gly-Phe-D-Pen]-OH) was antagonized by BNTX (7-benzylidenenaltrexone) and this subtype of the δ-opioid receptor was designated as the δ_1-opioid receptor (Portoghese et al., 1992; Sofuoglu et al., 1993). The analgesic effects of DSLET (Tyr-D-Ser-Gly-Phe-Leu-Thr) and deltorphin II (Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH_2) were blocked by naltriben and this subset was defined as the δ_2-opioid receptor (Sofuoglu et al., 1991; Takemori et al., 1992). The lack of antinociceptive tolerance between DPDPE and [D-Ala^2]deltorphin II provided further support for the existence of δ-receptor subtypes (Mattia et al., 1991).

Receptor binding studies performed with various ligands and tissues resulted in conflicting results, showing only one (Connor et al., 1997; Toll et al., 1997) or multiple (Fang et al., 1994; Kim et al., 2001) δ-opioid sites. Receptor autoradiography using proposed δ_1- and δ_2-selective agonists either has not revealed a discrete distribution for the two receptor subtype (Gouarderes et al., 1993) or has shown that the binding sites of [^{3}H]DPDPE and [^{3}H]DSLET displayed differences in some single anatomical structures (Hiller et al., 1996). Adenylyl cyclase measurements (Buzas et al., 1994; Olianas and Onali, 1995) and antisense mapping (Rossi et al., 1997; Standifer et al., 1994) were in accordance with the existence of opioid receptor subtypes.

Abbreviations: B_{max}, receptor density; BNTX, 7-benzylidenenaltrexone; DADLE, [D-Ala^2,D-Leu^5]enkephalin; deltorphin II, Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH_2; DOR-1, δ-opioid receptor; DOR-KO, δ-opioid receptor knock-out; DPDPE, Tyr-c[D-Pen-Gly-Phe-D-Pen]-OH; DSLET, Tyr-D-Ser-Gly-Phe-Leu-Thr; ED_{50}, the concentration of the agonist required to achieve 50% of the maximal stimulation; E_{max}, maximal stimulation; GDP, Guanosine 5′-diphosphate (sodium salt); GTP-γ-S-Li_4, Guanosine 5′-[γ-thio]triphosphate tetralithium salt; hDOR-CHO, Chinese hamster ovary cells stably transfected with the human δ-opioid receptors; IC_{50}, the concentration of the ligand required to achieve 50% inhibition; i.t., *intrathecal*; K_D, equilibrium dissociation constant; K_e, apparent antagonist affinity constant; K_i, inhibitory constant; Tic, 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid; TIPP, H-Tyr-Tic-Phe-Phe-OH; Tris, hexahydrate, Tris(hydroxymethyl)-aminomethane; [^{35}S]GTPγS, Guanosine-5′-*O*-(3-[^{35}S]thio)triphosphate.

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However, the pharmacologically defined subtypes have not been identified at a molecular level to date (Allouche et al., 2000; Zaki et al., 1996). Only one δ-opioid receptor gene (DOR-1) has been cloned from the amphibian *Rana pipiens* (Stevens et al., 2007), mouse (Zhu et al., 1999), rat (Fukuda et al., 1993) and human (Knapp et al., 1994) brain so far. It has been shown that the DOR-1 gene encodes both δ_1 and δ_2 subtypes (Zhu et al., 1999). Contrary, two δ-opioid receptor genes have been cloned from *zebrafish* (Barrallo et al., 1998; Pinal-Seoane et al., 2006), but they did not seem to correspond to the proposed δ-opioid receptor subtypes (Gonzalez-Nunez et al., 2007). Studies on the mRNA distribution of the cloned δ-opioid receptor also showed no evidence for the existence of δ-opioid receptor subtypes (Mansour et al., 1995). One splice variant has been revealed by mRNA analysis in mouse brain (Gavériaux-Ruff et al., 1997), but the existence of splice variant at the protein level remains to be demonstrated.

Recently, however, it was reported that the putative δ_1- and δ_2-agonists have opposing and synergist effects on ethanol consumption. Thereby the authors have reinforced the idea that δ_1- and δ_2-opioid receptors are distinct molecular targets (van Rijn and Whistler, 2009). Also recently, δ_1-opioid receptors have been implicated in reducing myocardial structure injury, while the δ_2-opioid receptors in raising the postischemic myocardial mechanical functions, both δ-opioid receptor subtypes attenuating myocardial injury by targeting the mitochondrial permeability transition pore (Zeng et al., 2010). These results suggest that the hypothetical δ-opioid receptor subtypes may participate in distinct physiological effects.

The availability of δ-opioid receptor antagonists with high potency and receptor selectivity may facilitate delineation of receptor types and subtypes. Using antagonists is advantageous, since the receptors might have different affinity states due to different G-protein coupling when agonist binding is studied. These distinct conformational states might be misinterpreted as receptor subtypes. Thereby, the aim of our work was to perform a comprehensive study on the possible existence of δ-opioid receptor subtypes both *in vivo* (antinociception) and *in vitro* (radioligand binding, receptor autoradiography, G-protein activation) in membranes of mouse brain and cells expressing a homogenous population of human δ-opioid receptors using a highly potent and selective δ-opioid antagonist, Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH. It is a derivative of TIPP (H-Tyr-Tic-Phe-Phe-OH; Tic: 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid), which represents the prototype of highly potent and selective, conformationally constrained δ-opioid antagonists (Schiller et al., 1992). By replacing each of the amino acids with corresponding β-methyl amino acid derivative in TIPP, it was found that Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH displayed the highest affinity and selectivity to δ-opioid receptors in receptor binding assays, and very high δ-antagonist potency in bioassays (Tourwé et al., 1998). Previously, we have radiolabeled the ligand and described its binding and signaling characteristics in rat brain and Chinese hamster ovary cells stably transfected with the human δ-opioid receptors (hDOR-CHO) (Birkas et al., 2008). We show in the present work that Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH behaved as a δ_1-like specific antagonist in the antinociceptive assay in mice. However, the putative δ_1- and δ_2-opioid receptors could not be unequivocally distinguished *in vitro*.

2. Material and methods

2.1. Chemicals

[^{3}H]Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH and [^{3}H]Ile^{5,6}-deltorphin II were synthesized and tritiated in the Isotope Laboratory of the Biological Research Center (Szeged, Hungary). Guanosine-5′-*O*-(3-[^{35}S]thio)triphosphate ([^{35}S]GTPγS) (37–42 TBq/mmol) was purchased from the Isotope Institute Ltd. (Budapest, Hungary). Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH, TIPP, Ile^{5,6}-deltorphin II and deltorphin II were synthesized in the Isotope Laboratory of the Biological Research Center (Szeged, Hungary) as described previously. DPDPE was from Bachem AG (Bubendorf, Germany). Naloxone, BNTX and naltriben were purchased from Tocris Bioscience (Ellisville, MO, USA). Guanosine 5′-diphosphate sodium salt (GDP), Guanosine 5′-[γ-thio]triphosphate tetralithium salt (GTP-γ-S-Li$_4$), magnesium chloride hexahydrate, Tris(hydroxymethyl)-aminomethane (Tris, free base), bacitracin, NaCl, ethylene-bis(oxyethylenenitrilo) tetraacetic acid (EGTA), Kodak Sigma Fixer, Kodak D-19 Developer and Kodak X-OMAT AR films were from Sigma-Aldrich Kft. (Budapest, Hungary). Bradford reagent and bovine serum albumin (BSA) were from Bio-Rad Laboratories (Hercules, CA, USA).

2.2. Analgesia measurement

.All procedures have been approved by the Institutional Animal Care and Use Committee of UMDNJ (Piscataway, NJ, USA). Mice (C57Bl/6J, both genders, 2–3 months old) were lightly anesthetized by isoflurane. *I.t.* lumbar puncture was performed using a modified version of the method of Hylden and Wilcox (Hylden and Wilcox, 1980) as reported (Porreca and Burks, 1983). All drugs were dissolved in physiological saline and administered to 7–16 mice/group. Animals were injected *intrathecally (i.t.)* with 5 μl saline or Tyr-Tic-(2*S*,3*R*)-β-MePhe-Phe-OH (10 μg, 13 nmole) immediately followed by 2 μl of either DPDPE (8 μg, 12 nmol), Ile^{5,6}-deltorphin II (15 μg, 19 nmole) or DAMGO (6 ng, 2 pmole). Analgesic latency was assessed by the tail-flick method 15 minutes later. The radiant heat tail-flick assay was performed as published using a light intensity that produced baseline latencies ranging from 2 to 3 s and a 10 s cut-off time (Zhu et al., 1999). The percent maximal possible effect (% MPE) was calculated using the formula: (measured value − baseline value)/(cut-off time − baseline value) × 100%. Group comparisons were performed by two-tailed *t*-test.

2.3. Mouse brain membrane preparation

Wild type mice (C57Bl/6J, male, 2-3 months old) were handled in accordance with the European Communities Council Directives (86/609/ECC) and the Hungarian Act for the Protection of Animals in Research (XXVIII.tv. Section 32). They were housed in a temperature- and light-controlled room. Lighting was ensured in a 12-h cycle, and food and water were available *ad libitum*. δ-opioid receptor knock-out, DOR-KO mice were generated by replacing exon 2 with a neomycin resistance cassette as published (Zhu et al., 1999). Whole brains were dissected and homogenized in 30 volumes (v/w) of ice-cold 50 mM Tris–HCl buffer (pH 7.4) with a teflon-glass Braun homogenizer operating at 1500 rpm. The homogenate was centrifuged at 20,000*g* for 25 min at 4 °C, the resulting supernatant was carefully discarded and the pellet was taken up in the original volume of Tris–HCl buffer. After homogenization with an all-glass Dounce, the homogenate was incubated at 37 °C for 30 min in a shaking water-bath. Centrifugation was then repeated as described above. The final pellet was suspended in 5 volumes of 50 mM Tris–HCl pH 7.4 buffer containing 0.32 M sucrose, frozen in liquid N_2 and stored at −80 °C. Prior to the experiments, an appropriate aliquot was melted, diluted with 5-fold Tris–HCl buffer and centrifuged at 20,000*g* for 25 min to remove sucrose. The protein content of the membrane preparation was determined by the method of Bradford, BSA being used as a standard (Bradford, 1976).

2.4. hDOR-CHO cell membranes

Membranes of Chinese Hamster Ovary, CHO cells stably transfected with the human δ-opioid receptors (hDOR-CHO, Malatynska

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