Opioid Receptor Binding Characteristics and Structure-Activity Studies of Novel Tetrapeptides in the TIPP (Tyr-Tic-Phe-Phe) Series

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Key Words

Opioid receptors · Delta antagonist peptides · Radioligand binding · [³⁵S]GTPγS binding · Rat brain, opioid receptors · Chinese hamster ovary (CHO) cell lines

Abstract

The development of the prototype synthetic δ-opioid receptor antagonist peptides TIPP [(H-Tyr-Tic-Phe-Phe-OH); Tic: tetrahydroisoquinoline-3-carboxylic acid] and TIPPψ (H-Tyr-ψTic-Phe-Phe-OH) by Schiller and co-workers was followed by extensive structure-activity relationship studies, leading to the emergence of numerous analogs that are of pharmacological interest. Eight novel diastereomeric compounds in this peptide family were designed, prepared, and tested biologically to gain structure-activity relationship information. The new multisubstituted tetrapeptide analogs contain both a 2',6'-dimethyltyrosine residue at the N-terminus and β-methyl-cyclohexylalanine at the third position as replacements for the original first tyrosine and the third phenylalanine, respectively. These derivatives wear either free acidic (–COOH) or amidated (–CONH₂) C-terminal. The potency and δ- versus μ-opioid receptor selec-

tivity were evaluated by in vitro radioreceptor-binding assays, while the intrinsic G-protein-activating efficacy of these analogs was tested in [³⁵S]GTPγS-binding assays using rat brain membranes or Chinese hamster ovary cells stably expressing μ- or δ-opioid receptors. The analogs showed δ-antagonist selectivity with differences regarding their isomeric forms, and these analogs containing a C-terminal carboxamide group displayed a mixed μ-agonist/δ-antagonist profile, thus they are expected to be safer analgesics with a low propensity to produce tolerance and physical dependence. These results constitute further examples of the influence of β-methyl substitution and C-terminal amidation on potency, selectivity, and signal transduction properties of TIPP-related peptides as well as they represent valuable pharmacological tools for opioid research.

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Introduction

Opioid receptors display biochemical and pharmacological heterogeneity and are divided into three individual types termed MOP- or μ-, DOP- or δ-, and KOP- or κ-receptors. The structural basis for opioid receptor mul-

tiplicity is clearly established by molecular cloning of cDNAs and genomic DNAs, encoding μ -, δ -, and κ -receptor proteins [1]. The heterogeneous populations of μ -, δ -, and κ -receptors are widely distributed, most importantly within the central nervous system. In opioid research and pharmacology, unambiguous distinction among the multiple receptors and effects is highly dependent upon the availability of selective ligands. A principal goal in the field is, therefore, the development of potent opioid agonists and antagonists of high specificity for each receptor type as well as ligands with mixed agonist-antagonist properties. Delta (δ) opioid ligands are especially important ligands in the study of narcotic addiction, since δ -receptor activation is not accompanied by severe signs of dependence in animal models [2, 3]. Selective ligands for δ -opioid receptors may also have therapeutic potential as immunosuppressants [4], and they offer a putative new approach to treat cocaine or alcohol addiction [5, 6]. A number of δ -receptor-selective antagonists have been described with diverse chemical structures, among them are peptide derivatives, modified epoxymorphinan alkaloid compounds, and others [7, 8].

Recently, a promising novel class of synthetic opioid peptide ligands with enhanced potency and selectivity toward δ -opioid receptors has been described. These peptides, termed TIPP analogs or TIPP peptide family [9], contain only aromatic residues, one of them being bicyclic 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (Tic). The prototype ligand in this group was Tyr-Tic-Phe-Phe (TIPP) which exhibited high affinity for the δ -opioid receptors in radioligand-binding experiments and behaved as potent δ -antagonist in the mouse vas deferens bioassay [10]. TIPP was designed with the aid of the concept of conformational restriction by introducing steric constraints into the native sequences. Enzymatic stability and δ -receptor potency were further enhanced by the formation of a pseudopeptide bond between the Tic² and Phe³ residues, resulting in a new molecule, Tyr-Tic ψ [CH₂-NH]Phe-Phe, named TIPP ψ [11]. Radiolabeling and biochemical characterizations of TIPP and TIPP ψ were performed in our laboratory [12, 13]. Replacement of Tyr¹ with 2',6'-dimethyltyrosine produced significant enhancement of δ -antagonist potency, but also a slight decrease in δ -receptor selectivity, as well as the μ -receptor affinity being increased [14]. Most interestingly, saturation of the Phe³ aromatic ring in TIPP and TIPP ψ , as achieved through substitution of cyclohexylalanine, led to a peptide with increased δ -antagonist property [15]. Subsequently, the Tyr-Tic dipeptide itself was shown to have an improved δ -antagonist character, and

the application of this motif in synthetic chemistry led to a new generation of ultrasensitive opioid peptides [16, 17]. A further outcome of these studies was the identification of potent and very selective dipeptide δ -agonists of the general formula H-Tyr-Tic-NH-arylalkyl which are of interest for drug development because of their low molecular weight and their lipophilic character. The conformational constraints imposed by the Tic residue eventuates the δ -antagonist properties. Addition of methyl groups at the second and sixth position of the tyrosine moiety forming 2,6-dimethyltyrosine (Dmt) and combining it with the Tic pharmacophore resulted in Dmt-Tic dipeptide with enhanced/remarkable δ -antagonist potency and unprecedented δ -receptor selectivity [18].

The use of β -methyl-substituted amino acids to constrain the conformational mobility of the side chain has been suggested by Hruby et al. [19, 20]. The effects of methylation of the β -carbon of a side chain on the biological properties of a peptide depend on the chiralities of the existing stereoisomers. Most interestingly, TIPP analogs containing a C-terminal carboxamide group displayed a mixed μ -agonist/ δ -antagonist profile, and thus were expected to be analgesics with a low propensity to produce tolerance and physical dependence [21], since previous observations clearly indicated that δ -opioid receptors play a major role in the development of morphine tolerance and dependence, suggesting the possibility of the combined use of a μ -type opioid analgesic and a δ -opioid antagonist in chronic pain treatment. Thus far important effects on receptor affinity, selectivity, and mixed μ -agonist/ δ -antagonist character have been observed in several peptide analogs [22].

The present structure-activity relationship study targeted the third aromatic side chain, i.e., phenylalanine (Phe³), in the TIPP structure, by replacing it with stereochemically different β -methyl-cyclohexylalanine (β MeCha) residues. Four possible isomers of novel TIPP-derived tetrapeptides, H-Dmt-Tic- β MeCha-Phe-OH, and all of their corresponding stereoisomers with amidated C-termini, H-Dmt-Tic- β MeCha-Phe-NH₂, were synthesized and tested in radioligand-binding assays and in [³⁵S]GTP γ S-binding experiments (see below) using rat brain membranes and Chinese hamster ovary (CHO) cell membranes transfected with human μ - or δ -opioid receptors. In this paper, we report that the tetrapeptide Dmt-Tic- β MeCha-Phe-OH (DIMCP) analogs are potent δ -selective opioid antagonists, whereas the C-terminally amidated analogs improved higher μ -receptor agonist properties. It is assumed that the β -methyl amino acid substitution at the third position along with C-terminal

amidation might result in a more potent mixed μ -agonist/ δ -antagonist property, leading to the design of new compounds with a diminished tolerance and dependence propensity.

Materials and Methods

Chemicals

[^3H][D-Ala²,N-MePhe⁴,Gly⁵-ol]enkephalin (DAMGO; 51 Ci/mmol) was purchased from DuPont de Nemours (Wilmington, Del., USA). [^3H]Ile^{5,6}-deltorphin-II (27 Ci/mmol) was prepared in our Isotope and Peptide Laboratory of the Biological Research Center (BRC Radiolab, Szeged, Hungary), as described [23]. Unlabeled DAMGO was purchased from Bachem (Bubendorf, Switzerland). Ile^{5,6}-deltorphin II and the TIPP analogs were synthesized in the BRC Radiolab or in the Department of Organic Chemistry of the Free University of Brussels (Belgium). Radiolabeled guanosine-5'-O-(3- γ [^{35}S]thio)triphosphate or [^{35}S]GTP γ S (37–41 TBq/mmol) was purchased from the Isotope Institute (Budapest, Hungary).

Peptide Synthesis

All peptides were prepared by solid-phase peptide synthesis on a Merrifield or MBHA resin by Boc chemistry. The commercially available Boc-Tyr, Boc-Pro, Boc-Phe, and Boc-erythro- β MeCha or Boc-threo- β MeCha new unnatural amino acids were prepared in our laboratories. Racemic erythro- β MeCha (2S,3S- β MeCha and 2R,3R- β MeCha) or threo- β MeCha (2S,3R- β MeCha and 2R,3S- β MeCha) were prepared from the corresponding erythro- or threo- β MePhe by hydrogenation at 60 psi H₂ and 50°C using Pt₂O catalyst for 20–24 h in a Parr shaker. Some of the results have been reported earlier [24]. Boc-protected racemic erythro- β MeCha or threo- β MeCha was prepared by known procedures. Epimeric peptides were separated by HPLC. Identification of the absolute configuration of the β MeCha residue in the peptides was performed after hydrolysis of the peptides followed by GITC derivatization and HPLC analysis and comparison with standards of known configurations [24]. Detailed description of the synthesis and chemistry of the peptides will be published elsewhere.

Animals

Inbred Wistar rats (weighing 250–300 g) were housed in the local animal house of the BRC (Szeged). The rats were kept in groups of 4 animals, allowed free access to food and water, and maintained on a 12/12-hour light/dark cycle until the time of sacrifice. The animals were treated according to the Directive 86/609/EEC of the European Union and the Hungarian Act for the Protection of Animals in Research (XXVIII.tv. 32 §).

Crude Rat Brain Membrane Preparation

A crude membrane fraction was prepared from Wistar rat forebrains according to a method of Pasternak, with small modifications [25]. Animals (2–3 months old) were decapitated, and the brains without cerebella were rapidly removed and washed several times with chilled 50 mM Tris-HCl buffer (pH 7.4). The brains were weighed and suspended in 30 v/w of brain tissue of the ice-cold buffer. Tissues were homogenized using a Braun Teflon-glass homogenizer (1,000 rpm/min, 10–15 strokes) and filtered through four layers of gauze to remove large aggregates. Additional buffer

was added to reach a final buffer volume/membrane pellet ratio of 30 (ml/g). After centrifugation with a Sorvall RC5C centrifuge (40,000 g at 4°C for 20 min), the resulting pellet was resuspended in fresh buffer (30 v/w) by using a vortex. The suspension was incubated at 37°C for 30 min to remove any endogenous opioids. Centrifugation was repeated under the same conditions as described above, and the final pellet was resuspended in 5 vol of 50 mM Tris-HCl buffer (pH 7.4) containing 0.32 M sucrose to give a final protein concentration of 3–4 mg/ml. The membranes were thawed and resuspended in 50 mM Tris-HCl buffer (pH 7.4) and centrifuged (40,000 g at 4°C for 20 min) to remove sucrose and used immediately in binding assays.

Membrane Preparation from CHO δ and CHO μ Cell Lines

CHO cells stably expressing the cloned human μ - and δ -opioid receptors were grown in Dulbecco's modified Eagle's medium (catalog No. D 7777; Sigma Chemical, St. Louis, Mo., USA) with F12 (Ham's) nutrient mixture with HEPES modification (cat. No. N 4138; Sigma Chemical) 1:1 (DMEM:F12 1:1) supplemented with 10% fetal calf serum, 2 mmol/l L-glutamine, 100 IU/ml penicillin, 100 μ g/ml streptomycin, and 0.4 mg/ml Geneticin G418 (cat. No. A 1720; Sigma Chemical). The cells were maintained in culture at 37°C in a CO₂ incubator with 5% CO₂. Membranes were prepared from confluent cultures. Cells were harvested and homogenized in phosphate-buffered saline for 15 s with a polytron homogenizer in an ice bath, followed by centrifugation at 1,000 g for 10 min. The pellet was resuspended in 20 ml TEM (0.1 mM Tris-HCl, 5 mM EDTA, 1 mM mercaptoethanol, pH 7.6) and centrifuged at 38,000 g for 20 min. The final pellet was reconstituted in a small volume of a 50-mM Tris-HCl buffer (pH 7.6) and stored in aliquots at –70°C until use.

Receptor-Binding Assays

The membrane suspensions from rat brain were incubated in polypropylene or glass assay tubes for 45 min at 35°C with the radioligand in a final volume of 1 ml and at a protein concentration of 0.3–0.5 mg/ml. Incubations were carried out in 50 mM Tris-HCl buffer (pH 7.4). Incubation was started by the addition of membrane suspension and terminated by rapid filtration through Whatman GF/C glass fiber filters using a Brandel M24R cell harvester. After three washings with 5-ml portions of ice-cold buffer (50 mM Tris-HCl), the filters were dried for 3 h at 37°C. The radioactivity was measured in a scintillation cocktail (Packard UltimaGOLD; PerkinElmer, Wellesley, Mass., USA), using a Packard (PerkinElmer) TriCarb 2300TR liquid scintillation analyzer with 58% counting efficiency. The potencies of competing ligands were determined by coinubation with 10^{–12} to 10^{–5} M freshly prepared solutions of the unlabeled drugs with 0.5–1 nM tritiated ligand. Nonspecific binding was defined as the radioactivity bound in the presence of 10 μ M unlabeled naloxone. All assays were performed in duplicate and repeated several times. Experimental data were analyzed by means of GraFit, a nonlinear least-squares curve fitting program or with GraphPad Prism [26]. Data were generally expressed as arithmetic mean values $\pm$ SEM of at least three repeated assays.

[^{35}S]GTP γ S Functional Assay

Rat brain membrane fractions ($\approx$ 10 μ g of protein/sample) were incubated at 30°C for 60 min in Tris-EGTA buffer (50 mM Tris-HCl, 1 mM EGTA, 3 mM MgCl₂, 100 μ M NaCl; pH 7.4) containing [^{35}S]GTP γ S (0.05 nM) and increasing concentrations (10^{–9} to

Table 1. Chemical structure of the newly developed DIMCP and DIMCPamide tetrapeptides

Sequence/composition	Compound	Chirality	Abbreviated name
<i>Reference compound</i>			
H-Tyr-Tic-Phe-Phe-OH			TIPP
<i>DIMCP analogs</i>			
H-Dmt-Tic-(2R,3S) β MeCha-Phe-OH	1	threo	(2R,3S) β MeCha-DIMCP
H-Dmt-Tic-(2S,3R) β MeCha-Phe-OH	2	threo	(2S,3R) β MeCha-DIMCP
H-Dmt-Tic-(2R,3R) β MeCha-Phe-OH	3	erythro	(2R,3R) β MeCha-DIMCP
H-Dmt-Tic-(2S,3S) β MeCha-Phe-OH	4	erythro	(2S,3S) β MeCha-DIMCP
<i>Amidated analogs; DIMCPamides</i>			
H-Dmt-Tic-(2R,3S) β MeCha-Phe-NH ₂	5	threo	(2R,3S) β -MeCha-DIMCPamide
H-Dmt-Tic-(2S,3R) β MeCha-Phe-NH ₂	6	threo	(2S,3R) β MeCha-DIMCPamide
H-Dmt-Tic-(2R,3R) β MeCha-Phe-NH ₂	7	erythro	(2R,3R) β MeCha-DIMCPamide
H-Dmt-Tic-(2S,3S) β MeCha-Phe-NH ₂	8	erythro	(2S,3S) β MeCha-DIMCPamide

10⁻⁵ M) of the compounds tested in the presence of 30 μ M guanosine 5'-diphosphate in a final volume of 1 ml, as described previously, with slight modifications [27]. Binding was determined either using CHO cell membranes expressing human μ - and δ -receptors ($\approx$ 10 μ g) or crude rat brain membranes ($\approx$ 10 μ g). Total binding was measured in the absence of the tested compounds, and nonspecific binding was determined in the presence of 10 μ M of unlabeled GTP γ S and subtracted from total binding to calculate the specific binding. The reaction was started by addition of [³⁵S]GTP γ S and terminated by vacuum filtration through Whatman GF/B filters with a Brandel M24R cell harvester, where bound and free [³⁵S]GTP γ S were separated. The filters were washed with 3 $\times$ 5 ml of ice-cold Tris-HCl buffer (50 mM, pH 7.4), and the bound radioactivity was detected in Packard UltimaGOLD scintillation cocktail with a Packard TriCarb 2300TR counter. Stimulation is given as percent of the specific [³⁵S]GTP γ S binding observed in the absence of receptor ligands (basal activity). Data were calculated from three independent experiments performed in triplicates and analyzed by sigmoid dose-response curve fit of the GraphPad Prism program [26].

Results

Eight novel TIPP-related tetrapeptide analogs were prepared, each containing 2',6'-dimethyltyrosine (Dmt¹) at the amino terminal end as well as β MeCha isomers at the third position. Four of the stereoisomers were composed of DIMCP, while four other analogs represented the corresponding C-terminally amidated tetrapeptides with the general structure of Dmt-Tic- β MeCha-Phe-NH₂ (DIMCPamide). Detailed structural information of the peptides, including Cahn, Ingold, and Prelog (CIP) configurations of the β MeCha stereoisomers, is given in table 1. Their biological activities were tested by in vitro

opioid receptor binding assays as well as [³⁵S]GTP γ S binding functional tests that measure the receptor-mediated activation of regulatory G-proteins.

Equilibrium Competition Binding Assays

Opioid affinities of Dmt-Tic- β MeCha-Phe analogs for δ - or μ -opioid receptors were assessed in radioligand-binding experiments using rat brain membrane fractions. Affinities for δ -opioid receptors were measured with the isoleucine-substituted highly δ -selective deltorphin-2 analog [³H]Ile^{5,6}deltorphin-II [23], while for μ -opioid receptors the affinities were determined by displacing the equilibrium binding of the prototype μ -specific radiolabeled enkephalin derivative, [³H]DAMGO. Heterologous displacement curves for the newly synthesized peptides competing reversibly with [³H]DAMGO binding are shown in figure 1, while K_i values are summarized in table 2. DIMCP and DIMCP amide analogs efficiently competed for the sites labeled by [³H]DAMGO, the latter ones being at least two times more potent as compared with those retaining unmodified C-termini. One exception was observed in the case of (2R,3S)DIMCP [Dmt-Tic-(2R,3S) β MeCha-Phe-OH] stereoisomer displacing [³H]DAMGO with the best affinity among all of the analogs tested affording a K_i value of 55 nM. Competition binding data for [³H]DAMGO were analyzed by one-site model [26], supposing that only a single set of binding sites is involved in the binding process.

Competition for the δ -agonist [³H]Ile^{5,6}deltorphin-II binding by the DIMCP analogs was more effective, exhibiting K_i values in the lower nanomolar range (table 2). Most of the [³H]Ile^{5,6}deltorphin-II heterologous competi-

Fig. 1. Competitive inhibition of [³H]DAMGO binding to rat brain membranes by unlabeled peptides. Membranes were incubated with 0.9–1.8 nM [³H]DAMGO for 45 min at 35°C in the presence of various (10⁻¹² to 10⁻⁵ M) concentrations of the competitors. Binding data (cpm) were analyzed by the GraphPad-Prism program. The data shown were normalized by the protein content and are expressed as percent specific binding. Mean values ± SEM of at least three independent experiments are shown, each performed in duplicate.

Table 2. Equilibrium competition binding data for diastereomeric β MeCha³-TIPP tetrapeptides in rat brain membranes

Ligands	Receptor binding (K_i , nM)		
	K_i^μ (n = 3)	K_i^δ (n = 3)	K_i^μ / K_i^δ
H-Tyr-Tic-Phe-Phe-OH (TIPP)	244 ± 13	32 ± 1	13.7
(2R,3S) β MeCha-DIMCP	55 ± 0.63	1.49 ± 0.14	36.9
(2S,3R) β MeCha-DIMCP	277 ± 47	2 ± 0.3	138.5
(2R,3R) β MeCha-DIMCP	575 ± 79.6	2 ± 0.58	287.5
(2S,3S) β MeCha-DIMCP	1,342 ± 149	0.48 ± 0.05	2,796
(2R,3S) β MeCha-DIMCPamide	202 ± 36.6	7 ± 1.3	28.8
(2S,3R) β MeCha-DIMCPamide	168 ± 10.9	4.1 ± 0.7	40.9
(2R,3R) β MeCha-DIMCPamide	289 ± 99.2	12.8 ± 2.6	22.5
(2S,3S) β MeCha-DIMCPamide	327 ± 106	4 ± 1.4	81.7

Receptor-binding data are presented as mean ± SEM of two or three numbers of repetitions from independent assays shown in parentheses. Heterologous displacement curves were analyzed by using a nonlinear regression algorithm (Prism). IC₅₀ values provided by the fitting procedure were then converted into equilibrium inhibitory constant (K_i) values, using the Cheng-Prusoff equation. K_i^μ inhibition constant measured by [³H]DAMGO. K_i^δ inhibition constant measured by [³H]Ile^{5,6}-deltorphin-II. The ratio of the affinities for δ - and μ -receptors (K_i^μ / K_i^δ) shows δ selectivity.

tion curves were adequately fitted according to the single-site binding model. Interestingly enough, in the case of two highly potent analogs, (2S,3R)DIMCP and (2R,3S)DIMCP, the binding processes were significantly better described (F-test comparison) by fitting data points according to a two-site model (fig. 2). Contrary to the conventional division of the differing affinity sites into ‘high’-

and ‘low’-affinity sites, both of the sites detected here were even of high affinity. The apparent presence of two binding components can indicate different affinity states, e.g., G-protein-coupled and G-protein-uncoupled forms, of the same receptor population. Another reason for the observed binding heterogeneity might be an interaction or cross talk among different receptor types or subtypes. The

Fig. 2. Competitive inhibition of [^3H]Ile 5,6 deltorphin-II binding to rat brain membranes by two DIMCP analogs. Samples were incubated with 0.9–2 nM [^3H]Ile 5,6 deltorphin-II for 45 min at 35°C in the presence of increasing (10^{-12} to 10^{-5} M) concentrations of either (2R,3S) DIMCP or (2S,3R) DIMCP. Curves were analyzed by both one-site and two-site competition models, and they were compared with an F test to obtain optimal fit. Data shown were normalized by the protein concentration and transformed into percent specific binding. The curves represent the results of the two-site fits. Mean values $\pm$ SEM of at least three experiments are shown, each performed in duplicate.

Fig. 3. Competitive inhibition of [^3H]Ile 5,6 deltorphin-II binding to rat brain membranes by two DIMCP analogs in the presence of G-protein reagents. Membranes were incubated with 0.9–2 nM [^3H]Ile 5,6 deltorphin-II for 45 min at 35°C in the presence of various (10^{-12} to 10^{-5} M) concentrations of either (2R,3S) DIMCP or (2S,3R) DIMCP and 10^{-6} M GppNHp in 10^{-4} M MgCl_2 . Curves obtained by one-site fit are shown. Mean values $\pm$ SEM of at least three experiments are given, each performed in duplicate.

latter seems not to be the case because of the high selectivity of the radioprobe. The existence of different affinity states is more likely, since the presence of specific G-protein reagents, such as the nonhydrolyzable GTP analog 5'-guanylylimidodiphosphate (GppNHp; 1 μM) and 100 μM MgCl_2 , were able to inhibit the ultrahigh-affinity sites by transforming the receptor populations into lower, although still very high-affinity states. Accordingly, dose-response curves are also shifted to the right and success-

fully analyzed by the single-site model assuming a homologous set of binding sites (fig. 3).

[^{35}S]GTP γS Binding Stimulation Assays

G-protein activation following the agonist occupation of the G-protein-coupled 7TM receptors can be determined by in vitro [^{35}S]GTP γS -binding assays [27, 28]. These tests are considered to be a measure of the functional activity of receptor ligands, primarily ago-

Fig. 4. Ligand-induced activation of [35 S]GTP γ S specific binding in δ_h CHO cell membranes stably expressing human δ -opioid receptors. Stimulation is given as a percentage of the nonstimulated (basal) level. The basal activity measured in the absence of receptor ligands is taken as 100% (basal = 'total binding' – 'nonspecific binding'). Nonspecific binding is determined in the presence of 10 μ M unlabeled GTP γ S. Incubations were carried out for 60 min at 30°C. Mean values $\pm$ SEM of at least three independent experiments are shown, each carried out in triplicate. Results of sigmoid dose-response curve fitting are plotted.

nists, to elicit signal transduction. Receptor-mediated G-protein activation is assayed by the radiolabeled [35 S]GTP γ S bound specifically to the G α subunit of G-proteins. GTP γ S is a nonhydrolyzable analog of GTP, capable of binding irreversibly to G-proteins, so they remain permanently activated upon receptor-stimulation by agonists [29, 30]. In order to evaluate the effects of DIMCP peptides, [35 S]GTP γ S-binding experiments were performed in various membrane preparations: crude membrane fractions of rat brain as well as membranes from cultured CHO cells transfected with either human δ -opioid or μ -opioid receptors. Activating potency (EC_{50} values) and efficacy (% maximal stimulation) of the peptide analogs were measured and compared to those of prototype peptide agonist ligands, such as the δ -agonist Ile^{5,6}deltorphin-II [20] and the μ -receptor-selective DAMGO [31]. Both in rat brain membrane preparations and in δ_h CHO cell membranes stably expressing the human δ -opioid receptors, Ile^{5,6}deltorphin-II caused a concentration-dependent increase in the binding of [35 S]GTP γ S over the basal level, affording an EC_{50} value of 37.2 ± 2.9 nM with a maximal stimulation of $302 \pm 48\%$ in δ_h CHO cell membranes (fig. 4). Studying the effects of the peptides in membranes from δ_h CHO cells, no increase in [35 S]GTP γ S binding was found in the presence of DIMCP analogs (fig. 1) or in the presence of the amidated derivatives (not shown). Instead, the concentration dependence of the peptides

is rather described by dipping lines, suggesting a tendency of inverse agonism (fig. 4).

CHO cell lines expressing human μ -opioid receptors, μ_h CHO, were also used to determine whether the novel analogs might carry a μ -agonist effect besides their δ -antagonist character. In the [35 S]GTP γ S-binding tests, all DIMCP amide derivatives displayed weak to moderate agonist properties (fig. 5). The EC_{50} values measuring the potency of the stimulatory effect ranged from 100 to 250 nM, depending on the configuration of the third β Me-Cha side chain. The maximal stimulation (E_{max} , efficacy) levels varied between 121 and 200% over the basal or non-stimulated activity (= 100%). The rank order of the efficacy was (2S,3R)DIMCPamide > (2R,3S)DIMCPamide > (2R,3R)DIMCPamide > (2S,3S)DIMCPamide. DIMCP derivatives with unchanged acidic C-termini also stimulated the [35 S]GTP γ S binding in μ_h CHO cell membranes, but with less potency of around 300 nM and weaker (110–170%) maximal stimulating effects (data not shown). The intrinsic agonist activity of the original TIPP and TIPP ψ δ -antagonists has previously been demonstrated in several cellular models [32, 33]. Regarding our data, the novel DIMCP ligands, especially the amidated analogs, possess at least partial agonist activities in μ -opioid receptor transfected CHO cells, thus behaving as mixed δ -antagonist/ μ -agonist ligands. Summarized E_{max} and EC_{50} values are shown in table 3.

Fig. 5. Ligand-induced activation of [³⁵S]GTPγS-specific binding in μ_hCHO cell membranes stably expressing human μ-opioid receptors. Stimulation is given as a percentage of the nonstimulated (basal) level. Basal activity (basal = 'total binding' – 'non-specific binding') is taken as 100%. Nonspecific binding is determined in the presence of 10 μM unlabeled GTPγS. Incubations were carried out for 60 min at 30°C. Mean values ± SEM of at least three independent experiments are shown, each carried out in triplicate. Results of sigmoid dose-response curve fitting are given.

Table 3. [³⁵S]GTPγS binding in response to TIPP analogs

Ligands	CHO- <i>hMOR</i> membranes	
	EC ₅₀ , nM	maximal stimulation, %
DAMGO	85.4 ± 11.5	280 ± 19
(2R,3S)βMeCha-DIMCP	299 ± 99	148.7 ± 7
(2S,3R)βMeCha-DIMCP	362.5 ± 83.5	169 ± 2.3
(2R,3R)βMeCha-DIMCP	447.5 ± 178.5	129.3 ± 1.2
(2S,3S)βMeCha-DIMCP	418.5 ± 205.5	111 ± 0.5
(2R,3S)βMeCha-DIMCPamide	102 ± 10	161 ± 9.5
(2S,3R)βMeCha-DIMCPamide	222 ± 4	199.7 ± 14
(2R,3R)βMeCha-DIMCPamide	478.5 ± 158.5	159.7 ± 14
(2S,3S)βMeCha-DIMCPamide	301.5 ± 42.5	121 ± 1.2

Agonist/antagonist-stimulated data are presented as mean ± SEM of two or three numbers of repetitions from independent assays. The parameters shown were obtained by non-linear regression analysis (GraphPad Prism 2.01). Data were converted to the percentage of [³⁵S]GTPγS binding in the presence of increasing drug concentrations as compared with basal binding in the absence of any drug.

[³⁵S]GTPγS-Binding Competition Assays

In the next set of experiments, the agonist-stimulated G-protein activation was inhibited by the DIMCP analogs in a competitive manner, indicating antagonistic abilities of the analogs in the presence of an activating full-agonist peptide. The potencies (ED₅₀ or IC₅₀ values) of putative antagonists in competing with the pure agonist can be measured by this method in a similar way as

in other conventional bioassays [28]. The DIMCP analogs effectively inhibited the δ-agonist-stimulated (10 μM Ile^{5,6}deltorphin-II) [³⁵S]GTPγS binding in a concentration-dependent manner using δ_hCHO cell membranes (fig. 6a). The C-terminally amidated analogs also decreased the agonist-induced stimulation of the nucleotide binding in both rat brain tissues (not shown) and δ_hCHO cell membranes (fig. 6b). The highest inhibiting potency

Fig. 6. Competitive inhibition of the agonist-stimulated [35 S]GTP γ S binding to δ_h CHO cell membranes stably expressing human δ -opioid receptors. Receptor-mediated G-protein activation was achieved by the addition of the δ -agonist Ile 5,6 deltorphin-II (10 μ M), and the subsequent 'displacement' was measured in the additional presence of increasing concentrations of competing DIMCP analogs (**a**) or DIMCPamide derivatives (**b**). Basal and stimulated activities and the nonspecific binding were calculated and plotted as in figures 4 and 5. Inhibition data were analyzed by the GraphPadPrism program using the 'one-site competition' option. Mean values $\pm$ SEM of at least three independent experiments are shown, each carried out in triplicate.

with an IC_{50} value of 2 ± 0.5 nM was obtained in the case of (2S,3R)DIMCPamide. Competing IC_{50} values for other compounds ranged from 50 to 100 nM. Taken together, in δ_h CHO cell membrane fractions, agonist-like direct

stimulatory effects of the peptides were not detected, while (2S,3R)DIMCP and (2S,3R)DIMCPamide analogs were able to antagonize the agonist action produced by Ile 5,6 deltorphin-II in these preparations.

Discussion

The development of the highly δ -receptor-selective TIPP tetrapeptide antagonists represents an important issue in the opioid peptide research, since both valuable experimental tools and potential therapeutic agents could be obtained. The current study examined the biochemical characteristics of some newly developed analogs within the TIPP family. These peptides were designed and synthesized, expecting enhanced selectivity for one particular receptor type or obtaining new compounds with improved μ -agonist/ δ -antagonist character. The original structure of TIPP, i.e., H-Tyr-Tic-Phe-Phe-OH, was further modified by systematic side-chain replacements with nonconventional amino acids. These unnatural amino acids were selected on the basis of their capability to possess well-defined side-chain conformations. The first tyrosine was, therefore, substituted for 2,6-dimethyl-tyrosine (Tyr¹→Dmt¹), while the third phenylalanine was replaced with the phenylalanine-mimetic β MeCha (Phe³→ β MeCha³). Since β MeCha exists in four different steric arrangements, each of the possible stereoisomers was built into the peptides, resulting in four analogs of H-Dmt-Tic- β MeCha-Phe-OH (DIMCP derivatives). The third type of modification was C-terminal amidation of the free carboxyl group that resulted in four DIMCP-amide ligands.

Detailed structure-activity studies of the peptides were undertaken in order to perceive the effects of steric and topographic constraints on the receptor-binding affinity and selectivity as well as on functional properties at the level of G-proteins. The ligands displayed good δ -affinities and moderate to weak μ -receptor potencies in binding studies. Overall, the amidated analogs exhibited slightly increased μ -receptor affinities as compared with those having an unmodified C-terminal. One notable exception was the (2R,3S)DIMPC, revealing the highest μ -affinity among all (table 2). Thus, the amidated peptides have the tendency of higher μ -receptor potencies, although their very good δ -binding activities were also retained. The highest δ -receptor affinities were obtained in the case of the erythro isomer (2S,3S)DIMCP (δ K_i 0.48 nM) and the threo analog (2R,3S)DIMCP (δ K_i 1.49 nM) against [³H]Ile^{5,6}deltorphin-II binding, but with substantially less affinity for μ -opioid sites labeled by [³H]DAMGO in rat brain preparations. Previous structure-affinity relationship studies in the TIPP family of δ -opioid receptor antagonist peptides have indicated the relative importance of the third position. The aromatic Phe³ residue was systematically replaced by halogenated

phenylalanine derivatives, such as *p*-Cl-Phe³ or *p*-I-Phe³, tryptophan, or homophenylalanine, and by nonaromatic alkyl amino acids, e.g., leucine, norvaline, isoleucine, or cyclohexylalanine [9]. The presence of a cyclic side chain at the third position was associated with higher δ -receptor potency, whereas the aromatic character turned out to be less important. Further studies of side chain methyl substitutions identified the (2S,3R) β -MePhe-TIPP analogs as potent δ -antagonists or δ -agonists, depending upon the acidic or amidated property of the C-terminal [22]. In (2S,3R) β -MePhe the *gauche*(−) and *trans* side-chain configurations are sterically allowed, while the *gauche*(+) configuration is strongly disfavored. In the current study, the (2S,3S) isomer displayed the highest affinity for the δ -receptor, whereas its affinity toward the μ -receptor was far lower. Consequently, the (2S,3S) β MeCha-DIMCP epimer represents the highest δ -receptor selectivity among all β MeCha-substituted tetrapeptides.

In functional studies performed on receptor-mediated G-protein activation, the novel DIMCP analogs exhibited different profiles in diverse in vitro systems. The native tissue preparations usually consist of mixed populations of cells, each expressing several types and different amounts of receptors. Thus, although DIMCP ligands did not significantly influence basal G-protein activity in rat brain homogenates, it is conceivable that a more sensitive assay would better distinguish the partial agonist, the inverse agonist, or the neutral antagonist characters of the ligands. Testing the effect of the peptides in rat brain membranes, none of the compounds activated [³⁵S]GTP γ S binding. Instead, a slight decrease of the basal activity was seen at higher concentrations. These observations indicated that the novel DIMCP analogs might be either neutral antagonists or, more likely, inverse agonists; further evidence for the inverse agonism was provided using δ_h CHO cell membranes stably expressing human δ -opioid receptors (fig. 4), where DIMCP analogs also behaved as inverse agonists. Similar findings have previously been demonstrated in digitonin-permeabilized GH₃DORT cells using the original TIPP peptide ligand [34, 35]. On the other hand, in μ_h CHO cells transfected with human μ -opioid receptors, the compounds dose dependently stimulated G-proteins (fig. 5), with maximal effects of 200% in the case of the (2S,3R)DIMCPamide isomer, thus becoming partial agonists in the μ -receptor-expressing system. By definition, partial agonists applied alone produced less maximal effects as compared with full agonists. At the μ -receptor sites, DAMGO is considered to be a full agonist, while deltorphins are full agonists at the δ -receptor sites. Both

neutral antagonists lacking intrinsic agonist activity and partial agonists also have the capability of antagonizing the stimulatory effects of a full agonist. In δ_h CHO cell membranes, DIMCP derivatives effectively inhibited the Ile^{5,6}deltorphin-II-induced stimulation of [³⁵S]GTP γ S binding (fig. 6). It is concluded that the novel tetrapeptides are neutral antagonists or mild inverse antagonists at the δ -receptors in functional tests, whereas their partial μ -agonist properties become evident in systems expressing solely μ -opioid receptors. In rat brain homogenates, where both μ - and δ -receptors are present, and in the absence of full agonists, the partial μ -agonist effect is apparently hidden, resulting in an overall antagonist-like behavior of the peptides.

The introduction of β -methyl substituents on each residue in TIPP can have important effects on receptor selectivity and on agonist/antagonist properties [22]. It has been reported that the dipeptides Tyr-Tic and especially Dmt-Tic themselves contain the necessary pharmacophore of the δ -antagonist effects [36, 37]. Although it was shown that a single β -methyl substituent does not constrain dramatically the conformational mobility of aromatic amino acids [38], the use of these topographically constrained amino acids remains important in mediating different biological activities. The introduction of a saturated cyclohexylalanine residue mimicking the phenylalanine side chain into the third position (Phe³→Cha³ replacement) resulted in substantially increased δ -antagonist potency and δ -receptor selectivity [15]. The present study demonstrated that the replacement of Phe³ with another isoster but nonaromatic amino acid, β MeCha, can also affect the biochemical features of the analogs. In such small peptides the side chains obviously exhibit considerable conformational mobility, so understanding the exact three-dimensional structural arrangements of the

peptides which constitute the μ - or δ -pharmacophore of the TIPP family upon receptor recognition remains a challenge. The use of β -methyl amino acids to restrict the conformational flexibility of certain side chains [19] is one of the reasonable approaches in exploring the fine-structural elements of ligand-receptor interactions. Since the results of such β -methyl substitutions are not simply interpreted in terms of topographical requirements, further studies including nuclear magnetic resonance investigations of the analogs and molecular simulation modeling are necessary to gain more precise insight into the bioactive conformations of receptor-interacting peptides.

In summary, the newly synthesized DIMCP analogs have a high affinity in [³H]Ile^{5,6}deltorphin-II-binding assays and effectively stimulate the G-proteins in μ_h CHO cell membranes expressing human μ -opioid receptors, while in δ_h CHO cell membranes expressing human δ -opioid, the analogs exhibit neutral antagonist or weak inverse agonist profiles. Our results indicate that the novel DIMCP compounds are potent, selective δ -antagonist ligands in rat brain preparations, and they mediate mixed μ -agonist/ δ -antagonist properties in cell membrane preparations *in vitro*. Thus, these novel tetrapeptide opioid ligands may serve as useful pharmacological and therapeutic agents, and they can represent valuable experimental tools in research and probably in medicine.

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