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Receptor binding and biological activity of the dermorphin analog Tyr-D-Arg^2-Phe-Sar (TAPS)

Stefan Vonhof [a,b,*], Frank C. Barone [c], William J. Price [c], Pirkko Paakkari [b], James H. Millison [b], Giora Z. Feuerstein [c], Anna-Leena K. Sirén [b,d]

[a] *Department of Cardiology and Pulmonology, Zentrum Innere Medizin, University of Göttingen, Robert-Koch-Str. 40, 37075 Göttingen, Germany*
[b] *Department of Neurology, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Road, Bethesda, MD 20814, USA*
[c] *Department of Cardiovascular Pharmacology, SmithKline Beecham, King of Prussia, PA 19406, USA*
[d] *Max-Planck-Institute for Experimental Medicine, Hermann-Rein-Str. 3, 37075 Göttingen, Germany*

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Abstract

The binding of Tyr-D-Arg^2-Phe-sarcosine(Sar)^4 (TAPS), a proposed μ-opioid receptor-selective tetrapeptide analog of dermorphin to opioid receptors, was studied using selective binding assays for subtypes of μ-, δ- and κ-opioid receptors. Subtype specific μ-opioid receptor binding was further characterized in the presence of sodium and guanosine nucleotides and the activity of TAPS in isolated guinea pig ileum was compared to other μ-opioid receptor-selective ligands. Further, the antinociceptive properties of TAPS following intrathecal (i.t.) administration in rats, as a model of spinal antinociception, were evaluated. The K_i-values for TAPS at the μ_1- and μ_2-opioid receptor sites were 0.4 and 1.3 nM, respectively, suggesting high affinity binding to μ-opioid receptor binding sites with an increased selectivity to μ_1-opioid receptor sites. The attenuated reduction of TAPS binding at the μ_2-opioid receptor subtype in the presence of the stable guanosintriphosphate analog 5′-guanylylimidodiphosphate and sodium suggests a potential partial antagonist mode of action at this site. © 2001 Published by Elsevier Science B.V.

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1. Introduction

Tyr-D-Arg^2-Phe-sarcosine^4 (TAPS) is a μ-opioid receptor-selective tetrapeptide analog of dermorphin (Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2), a heptapeptide isolated from the skin of *Phyllomedusa sauvagei* (De Castiglione and Rossi, 1985). Dermorphin selectively binds to μ-opioid receptors (De Castiglione and Rossi, 1985; Krumins, 1987) and displays potent opiate-like antinociceptive properties (Broccardo et al., 1981; Stevens and Yaksh, 1989), catalepsy and respiratory depression (Paakkari and Feuerstein, 1988; Paakkari et al., 1989). The N-terminal tetrapeptide of dermorphin constitutes the minimally required sequence in order to retain its opioid activity. Although this fragment is less potent than the parent heptapeptide (Broccardo et al., 1981; Salvadori et al., 1982), substitution of the D-Ala^2 residue with D-Arg and Gly^4 with sarcosine (Sar) greatly enhances the potency of the tetrapeptide. Thus, in antinociceptive tests TAPS was about six times as potent as dermorphin (Sasaki et al., 1984; De Castiglione and Rossi, 1985; Sato et al., 1987). The D-Arg^2,Sar^4-substitutions also increased binding of dimeric dermorphin analogues to μ-opioid receptors while binding to δ-opioid receptors was decreased, yielding a very high μ/δ-selectivity ratio (Lazarus et al., 1989). Although TAPS exerted an enhanced antinociceptive potency compared to morphine (Sato et al., 1987; Paakkari et al., 1993) and induced catalepsy, TAPS stimulated respiration at equianalgetic doses compared to morphine, when injected into the cerebral ventricles (i.c.v.) (Paakkari et al., 1993).

Based on the pharmacological profiles of the opioid antagonists naloxonazine and naloxazone, the existence of at least two μ-opioid receptor subtypes, μ_1 and μ_2, has been proposed earlier (Reisine and Pasternak, 1996). These subtypes exhibit a differential distribution throughout the central nervous system (Moskowitz and Goodman, 1985; Goodman and Pasternak, 1985). As for TAPS, the

* Corresponding author. Department of Cardiology and Pulmonology, Zentrum Innere Medizin, Robert-Koch-Str. 40, 37075 Göttingen, Germany. Tel.: +49-551-398-892; fax: +49-551-398-896.
E-mail address: svonhof@gwdg.de (S. Vonhof).

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antinociceptive, cataleptic and respiratory effects following i.c.v. administration were antagonized by naloxonazine, suggesting a μ_1-opioid receptor subtype-related mechanism (Paakkari et al., 1993). A strong μ_1-opioid receptor agonist activity of TAPS was further supported by studies demonstrating that the TAPS-induced inhibition of adenylate cyclase activity in rat caudate putamen and nucleus accumbens was antagonized by naloxonazine (Izenwasser et al., 1993). In accordance, the TAPS-induced inhibition of potassium-induced release of norepinephrine in rat cortex was equally attenuated in the presence of naloxonazine (Kim and Cox, 1993).

The purpose of the present study was to examine whether the strong μ_1-opioid receptor agonist activity in vivo and in vitro of TAPS could also be confirmed in receptor binding studies. Therefore, the binding of TAPS to various opioid receptors was studied using established μ_1- and μ_2- (Clark et al., 1988), δ- (Clark et al., 1986), κ_1- and κ_2-opioid receptor-specific binding assays (Zukin et al., 1988). Further, the binding of [^{3}H]TAPS to opioid receptors was characterized. Since preliminary binding data showed binding of TAPS at the μ_2-opioid receptor binding site, the in vitro activities of TAPS and of the μ-opioid receptor agonist [D-Ala^2,MePhe^4,Gly(ol)^5]enkephalin (DAMGO) in the guinea pig ileum, a proposed model for μ_2-opioid receptor subtype-mediated action (Gintzler and Pasternak, 1983) were characterized. Further, previous studies by Pasternak et al. have suggested that morphine antinociception after i.c.v. administration is mediated via μ_1-opioid receptor activation whereas μ_2-opioid receptors appear to mediate morphine antinociception at the spinal level (Ling and Pasternak, 1982; Heyman et al., 1988; Paul and Pasternak, 1988). Therefore, the antinociceptive and cataleptic effects were evaluated following intrathecal (i.t.) administration of TAPS. In accordance to our previously published results regarding respiratory effects after i.c.v. administration (Paakkari et al., 1993), we also measured the effects of TAPS on ventilation, when given i.t.

2. Materials and methods

2.1. Receptor binding assays

2.1.1. μ_1 / μ_2-Opioid receptor subtype-specific assays

The assay conditions were adopted without modification from established μ_1- and μ_2-opioid receptor binding assays described in the literature (Clark et al., 1988). Briefly, calf medial thalami were homogenized in 35 volumes of Tris-buffer and centrifuged at $49\,000 \times g$ for 40 min. The pellet was resuspended in 0.32 M sucrose (166.7 mg/ml) and frozen at −70°C for a maximum of 4 weeks. Duplicate samples of bovine thalamic membranes were incubated in potassium phosphate buffer (50 mM, pH 7.0) with 5 mM $MgSO_4$ (15 mg wet weight of tissue/ml, total incubation volume 3 ml) for 150 min at 25°C. The μ_1-opioid receptor subtype-selective saturation assay was performed using [^{3}H][D-Ala^2,D-Leu^5]enkephalin (DADLE), an enkephalin analog with poor affinity to μ_2-opioid receptors (Clark et al., 1988) in the presence of the δ-opioid receptor-selective ligand [D-Pen^2,D-Pen^5]enkephalin (DPDPE, 10 nM) which exhibits low affinity for μ_1-opioid receptor binding sites (Clark et al., 1986). In competition assays a 0.9 nM concentration of radioligand was used. The μ_2-opioid receptor subtype-specific saturation assays were performed using the highly μ-opioid receptor-selective agonist [^{3}H]DAMGO in the presence of [D-Ser^2,Leu^5]enkephalin-Thr^6 (DSTLE, 5 nM), an opioid analog with high affinity to μ_1- and δ-opioid binding sites (Clark et al., 1988). Competition assays were performed using a 1.2 nM concentration of the radioligand. The effect of guanyl nucleotides and sodium on μ-opioid receptor binding was studied in the presence of 10 μM of the stable guanosintriphosphate analog, 5′-guanylylimidodiphosphate (Gpp(NH)p) and sodium chloride (100 mM). As the μ-opioid receptors are coupled to an inhibitory G-protein, inclusion of guanyl nucleotides in the assay reduces the affinity of μ-opioid receptor agonists while antagonist binding is only slightly altered (Childers and Snyder, 1978; Puttfarcken et al., 1988; Werling et al., 1988). Additionally, sodium chloride enhances the inhibitory effect of guanyl nucleotides on μ-opioid receptor agonist binding (Childers and Snyder, 1978).

2.1.2. δ-Opioid receptor-specific assays

Frontal cortex tissue of calf brain was processed as described above. In saturation assays, duplicate samples of bovine cortical membranes were incubated in potassium phosphate buffer (50 mM, pH 7.0) with 5 mM $MgSO_4$ (10 mg tissue wet weight/ml, total incubation volume 3 ml) with [^{3}H]DPDPE for 90 min at 25°C. A 0.7 nM concentration of the radioligand was used in competition assays.

2.1.3. κ_1-Opioid receptor-specific assays

κ_1-Opioid receptor binding was assessed in homogenates of guinea pig cerebellum as described earlier by Zukin et al. (1988). The tissue was processed as described above. Saturation assays of tritiated (5α,7α,8β)-(+)-*N*-methyl-*N*-7-(1-pyrrolidinyl)-1-oxaspirol(4,5)dec-8-ybenzeneacetamide ([^{3}H]U69593) were performed in 50 mM Tris–HCl buffer (pH 7.5) (15 mg tissue wet weight/ml) for 60 min at 25°C. In competition assays, a 0.4 nM radioligand concentration was used.

2.1.4. κ_2-Opioid receptor-specific assays

Binding was determined in homogenates of whole rat forebrain which were prepared fresh for each assay as described earlier by Zukin et al. (1988). For saturation assays, rat brain membranes (15 mg tissue wet weight/ml) were incubated with tritiated ethylketocyclazocine ([^{3}H]EKC) in the presence of 100 nM DAMGO, 100 nM

DADLE and 1 mM U69593 in 50 mM Tris–HCl (pH 7.5) buffer for 150 min at 25°C. Competition assays were done using 2.7 nM [^{3}H]EKC.

2.1.5. Binding of [^{3}H]TAPS

TAPS binding was further characterized in bovine thalamic homogenates using [^{3}H]TAPS. The effect of guanyl nucleotides and sodium on TAPS binding was studied in the presence of 10 μM Gpp(NH)p and sodium chloride (100 mM). The incubation was stopped with three rinses of ice-cold potassium phosphate buffer and rapid filtration through Whatman GF/B filter paper using a Brandel Cell Harvester (Brandel, Gaithersburg, MD). In all assays, non-specific binding was determined in the presence of levallorphan (1 μM). Only specific binding (approximately 70% of total binding) is reported.

2.2. Activity of TAPS in isolated guinea pig ileum

Male guinea pigs (350–500 g, Taconic farms) were sacrificed by cervical dislocation and ileal segments of 1.5 cm length were excised. The tissue was washed with Krebs–Henseleit solution containing 118.2 mM NaCl, 2.52 mM $CaCl_2$, 1.18 mM KH_2PO_4, 4.69 mM KCl, 2.41 mM $MgSO_4$, 11.65 mM dextrose, and 25,0 mM $NaHCO_3$. The ileal segments were then suspended in jacketed organ baths, filled with oxygenated Krebs–Henseleit solution maintained at 37°C and were affixed to isometric force transducers (FT 0.03, Grass Instrument) using a single suture of 3.0 nylon on each end of the segment. Four segments were removed from each animal. The *n* in each treatment/drug concentration group reflects the actual number of segments from different guinea pigs. Tissue tension was monitored on an eight-channel polygraph (Dynagraph recorder R611). Initial tension (1 g) was applied to each segment and tissues were allowed to equilibrate over 45–60 min before testing. A baseline period (30 min) of transmural electrical stimulation was determined using parameters (single pulses at 80 V, 0.1 ms, 0.1 Hz, Grass Instruments, S88 Stimulator) that produced submaximal, consistent contractions, ranging from 3 to 5 g in magnitude through activation of two platinum wire electrodes, one located along the inside of the tissue segment and the other located along the outside. Three consistent responses, which occurred after the addition of increasing agonist concentrations were averaged and normalized as a percentage of the baseline period contractions. In studies using opioid antagonists, the tissues were pretreated with effective antagonist concentrations for 30 (nor-binaltorphimine, 20 nM) and 60 min (naloxonazine, 50 nM). After pretreatment with naloxonazine, the ileal segments were washed for 60 min with Krebs–Henseleit buffer, exchanging the buffer solution at 10 min intervals in order to remove reversibly bound naloxonazine. Preliminary experiments showed a potent and complete antagonism by naloxonazine to all agonists used when it was not removed from the tissue bath through the washing steps mentioned above (data not shown). These washing procedures were similar to the method described by Gintzler and Pasternak (1983), showing a 95% recovery of morphine action in the guinea pig ileum assay at the μ_2-opioid receptor binding site, which is occupied by naloxonazine in a competitive mode of action. Thus, the remaining antagonist effect was due to the non-competitive antagonist action of naloxonazine at the μ_1-opioid receptor binding site. The dose of 50 nM was derived from receptor binding studies showing that naloxonazine effectively occupies μ_1-opioid receptor binding sites at this concentration (Gintzler and Pasternak, 1983). Antagonist pretreatment did not affect stimulator-induced responses. Three consistent responses to increasing concentrations of the same agonist in the presence of antagonist were normalized as described above.

2.3. Antinociception and catalepsy

Male Sprague–Dawley rats (275–350 g, Taconic Farms, Germantown, NY) were used. Under halothane anesthesia (4% in 100% oxygen) the atlanto-occipital membrane was exposed and an intrathecal (i.t.) PE-10 catheter (5 cm) was inserted into the subarachnoid space. The catheter was secured with a 3.0 nylon thread. In addition, a PE-50 catheter was inserted into the jugular vein and exteriorized at the nape of the neck. The animals were allowed to recover for 48 h prior to the experiment.

For dose–response studies, TAPS was injected i.t. at doses of 10, 30 or 100 pmol/rat. In studies using receptor-specific antagonists, naloxonazine (10 or 20 mg/kg) (Ling et al., 1986) was injected intravenously (i.v.) or i.t. 24 h before TAPS administration. Naloxone (5 mg/kg) and naltrindole (5 mg/kg) were administered intravenously 5 and 15 min before TAPS, respectively. Nor-binaltorphimine (1, 3 or 10 nmol) was injected i.t. 15 min before TAPS. Antinociception was measured using the radiant tail-flick latency method on a tail-flick apparatus (Socrel, Milano, Italy). The data are presented as percent of the maximal possible effect (%MPE) as described elsewhere (Vonhof and Siren, 1991). A maximal exposure of 12 s was chosen as cut-off in order to limit heat-induced tissue damage. Catalepsy was assessed by placing the front limbs of the rat over a 10-cm high horizontal bar and measuring the time that the animal maintained that posture. A cataleptic state was defined if the rat remained on the bar as positioned before for a minimum of 60 s. The righting reflex following i.t. administration of TAPS was studied by turning the animals on their backs. The righting reflex was determined to be preserved when the animals made attempts to regain their posture within 10 s.

2.4. Measurement of respiration

Respiration rate and relative respiratory tidal volume were monitored using the Oxymax 85 system (Columbus

Instruments, Columbus, OH) as described previously (Paakkari et al., 1993). This computerized recording system consists of two plexiglas test chambers and one reference chamber with a constant flow of room air of 2 l/min each. The respiration rate is determined based on the frequency (respirations/min) of pressure changes due to the ventilatory movements of the rat's thorax. The tidal volume is recorded as an integral of pulses which is proportional to the amplitude of the pressure changes and compared to the count in the first sample measure of the experiment. The tidal volume is therefore expressed as relative tidal volume in arbitrary units. The relative ventilatory minute volume is calculated as the arithmetical product of respiration rate and relative tidal volume. The accuracy of the respiration rate recording is ±1%. The respiratory data are gathered at 2-min intervals.

The rats were prepared as described above. Initially, the animals were allowed to accommodate for 60 min before the drugs were given as described above. Five consecutive recordings of the aforementioned ventilatory parameters during the 10-min period immediately preceding the first drug administration were averaged and defined as the baseline conditions. In order to minimize the effect on ventilation caused by handling of the animals during the injection procedures, the data were evaluated starting 30 min following the drug injection. For comparison, areas under the curves for changes in respiration rate, relative tidal volume and minute volume were determined after i.t. administration of TAPS vs. control.

2.5. Drugs

[^{3}H]DAMGO (32.3 Ci/mmol), [^{3}H]DADLE (47.2 Ci/mmol), [^{3}H]DPDPE (35 Ci/mmol) and [^{3}H]EKC (28.1 Ci/mmol) were purchased from New England Nuclear (Boston, MA). [^{3}H]U69593 (54 Ci/mmol) and custom tritiated [^{3}H]TAPS (47.3 Ci/mmol) were obtained from Amersham (Arlington Heights, IL). 5′ guanylylimidodiphosphate (Gpp(NH)p) was obtained from Sigma (St. Louis, MO). The unlabelled opioid peptides were purchased from Peninsula (Belmont, CA), naloxone, naloxonazine, EKC, *trans*-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl) cyclohexyl] benzene acetamide (U50488), (5α, 7α,8β)-(+)-*N*-methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspirol-[4,5]dec-8-y-]-benzeneacetamide (U69593) from Research Biochemicals (Natick, MA). (−)-(1*R*,5*R*,9*R*,2″*S*)-5,9-dimethyl-2′-hydroxy-2-tetrahydrofurfuryl-6,7-benzomorphan D-tartrate (MR2034) was a gift from Boehringer Ingelheim (Ingelheim am Rhein, F.R.G.). Nor-binaltorphimine and naltrindole were obtained from the Research Technology Branch of the National Institute of Drug Abuse (Rockville, MD). In the binding assays, the drugs were dissolved in the appropriate buffer and for the in vitro assays all drugs were dissolved in 0.9% NaCl. Naloxonazine was solubilized in buffer containing glacial acetic acid. The final concentration of acetic acid in the solution was 2%.

2.6. Data processing and statistics

The receptor binding data were analyzed using the LIGAND program to calculate dissociation and inhibitor constants (K_d and K_i) and maximal receptor density (B_{max}) (Munson and Rodbard, 1980). For statistical analysis, the CSS: STATISTICA software package (StatSoft, Tulsa, OK) was used. Results are expressed as means ± S.E.M. for the given number of experiments. For statistical analysis of data with equal variances, the analysis of variance (ANOVA) or ANOVA for repeated measures followed by Tukey's multiple comparison test or Student's *t*-test were used. The Kruskal–Wallis nonparametric ANOVA and the Mann–Whitney *U*-test were used for groups with unequal variances. The normalized data derived from experiments on guinea pig ileum are presented as the mean percent inhibition of the basal contractile response. In addition, because of the nature of the inhibition curves (i.e., typically only 50 % of the contraction could be inhibited with any agonist), the IC_{20} values were determined from the regression line using computerized fitting algorythms for each agonist concentration–response curve in the presence or absence of antagonist.

3. Results

3.1. Receptor binding studies

The K_d for [^{3}H]DADLE in the μ_1-opioid receptor subtype-selective assay was 0.9 nM and the maximal receptor density (B_{max}) was 2.9 ± 0.2 fmol/mg tissue wet weight (ww). The corresponding values for [^{3}H]DAMGO in the μ_2-opioid receptor subtype-selective assay were 1.4 nM and 3.6 ± 0.3 fmol/mg ww. Additionally, Scatchard plots were linear in both assay types indicating the involvement of a single binding site in each assay setup. These K_d and B_{max} values are comparable to those reported in the literature under similar assay conditions (Clark et al., 1988). Saturation binding of [^{3}H]DPDPE at δ-opioid receptor sites revealed a K_d of 0.7 ± 0.1 nM (B_{max} = 1.4 ± 0.1 fmol/mg ww). The affinities of [^{3}H]U69593 and [^{3}H]EKC for κ_1- and κ_2-opioid receptor binding sites were 0.4 ± 0.1 nM (B_{max} = 1.6 ± 0.1 fmol/mg ww) and 2.7 ± 0.8 nM (B_{max} = 0.9 ± 0.3 fmol/mg ww), respectively. Competition studies with unlabeled TAPS against [^{3}H]DADLE or [^{3}H]DAMGO demonstrated that TAPS bound with high affinity to both μ-opioid receptor subtypes. The K_i of TAPS at the μ_1-site was 2-fold higher as compared to its affinity to the μ_2-opioid receptor site (Table 1). In contrast the affinity of TAPS at δ- and κ-opioid receptor binding sites was at least two orders of magnitude lower as compared to its affinity at μ-opioid receptor sites. [^{3}H]TAPS displayed high affinity binding to thalamic membranes with an apparent K_d of 0.3 ± 0.1 and B_{max} of 2.8 ± 0.1 fmol/mg ww.

Table 1
Binding characteristics of TAPS

Radioligand	Site	K_i (nM)	B_{max} (fmol/mg)	$R_{x/\mu}$
[^{3}H]DAMGO	μ	0.5 ± 0.1	1.6 ± 0.4	1.0
[^{3}H]DADLE	μ, δ	0.8 ± 0.2	1.1 ± 0.4	1.6
[^{3}H]DADLE (+DPDPE)	μ_1	0.4 ± 0.1	1.5 ± 0.2	0.8
[^{3}H]DAMGO (+DSTLE)	μ_2	1.3 ± 0.1	1.9 ± 0.2	2.6
[^{3}H]DPDPE	δ	562.0 ± 88.7	0.7 ± 0.1	1124
[^{3}H]U69593	κ_1	> 10 000	n.d.	> 20 000
[^{3}H]EKC (+DAMGO, DADLE, U69593)	κ_2	107.1 ± 23.1	1.1 ± 0.1	214.2

Competition binding was performed in several opioid receptor subtype-specific assays. Inhibitor constants (K_i) and maximal binding density (B_{max}) were determined from Eadie–Hofstee plots as described. $R_{x/\mu}$ denotes the relative affinity compared to competition against [^{3}H]DAMGO. n.d. = not determined. The assays were performed in replicates of at least three.

[^{3}H]TAPS saturation in the presence of 5 nM DSTLE in order to achieve similar conditions as in the μ_2-opioid receptor subtype-specific assay using [^{3}H]DAMGO showed a K_d of 1.3 ± 0.1 nM (Fig. 1).

Since TAPS displayed high affinity to both μ_1- and μ_2-opioid receptor subtypes but our in vivo data suggested that TAPS may exhibit strong μ_1-opioid receptor agonist activity and reduced activity at the μ_2-opioid receptor site, binding of TAPS at the μ_1- and μ_2-opioid receptor sites was studied in the presence of sodium and the stable GTP analog, Gpp(NH)p. Self-displacement of DADLE and DAMGO in the presence of Gpp(NH)p and sodium demonstrated that the agonist affinities were severely reduced in both the μ_1-opioid receptor subtype-selective and μ_2-opioid receptor subtype-selective assays. The K_d for DADLE increased 4.8-fold and the K_d for DAMGO was increased 10.5-fold (Table 2). Addition of Gpp(NH)p and sodium in the incubation medium increased the K_i for TAPS in the μ_1-opioid receptor-selective assay 12.5-fold and 3.3-fold in the μ_2-opioid receptor-selective assay (Table 2). The affinity changes of the κ-opioid receptor agonists and proposed μ_2-opioid receptor antagonists EKC and MR2034 (Wood et al., 1982) in the μ_2-opioid receptor subtype-selective assay were similar to TAPS, the K_i for EKC increased 5-fold and that of MR2034 increased 3.6-fold when Gpp(NH)p and sodium were present. The affinity of the full antagonist, naloxone, was not affected by addition of sodium and Gpp(NH)p. Saturation of [^{3}H]TAPS in the presence of sodium and Gpp(NH)p resulted in a shift of the apparent K_d to 9.0 ± 1.2 nM, representing a 6.4-fold change when compared to controls.

3.2. Activity of TAPS on the isolated guinea pig ileum

The ability of TAPS to inhibit electrically induced contractions of guinea pig ileum was comparable to that of DAMGO (Table 3 and Fig. 2A). These effects were completely inhibited by naloxone (10 nM, data not shown). In order to compare the putative μ_2-opioid receptor subtype-mediated effects on guinea pig ileum, the ileal preparations were first treated with naloxonazine (50 nM) in order to selectively block the μ_1-opioid receptor subtype-mediated

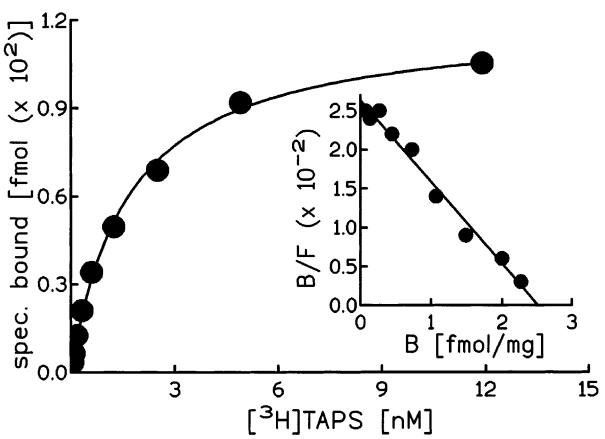

Fig. 1. Saturation experiments on crude homogenates of bovine medial thalamus using [^{3}H]TAPS including 5 nM DSTLE. Nonspecific binding was determined in the presence of 1 μM levallorphan. Only specific binding is shown ($n = 3$). The inset shows Scatchard analysis of the binding data. The K_d was 1.3 ± 0.1 nM and maximal receptor density (B_{max}) was 2.3 ± 0.2 fmol/mg tissue wet weight (ww). Regression analysis showed a significant fit involving a single binding site. B = bound radioligand, F = free radioligand.

Table 2
Effect of sodium and Gpp(NH)p on the affinities of DAMGO, DADLE and TAPS in μ_1- and μ_2-opioid receptor subtype-specific binding assays. The assays were performed in replicates of at least three. The last column (ratio) shows the change in affinity in the presence of sodium and Gpp(NH)p

Site	Competitor	NaCl + Gpp(NH)p	K_d (nM)	Ratio
μ_1	DADLE	−	2.0 ± 0.1	4.8
		+	9.6 ± 2.2	
μ_1	TAPS	−	0.7 ± 0.1	12.6
		+	8.8 ± 4.6	
μ_2	DAMGO	−	1.5 ± 0.1	10.5
		+	15.8 ± 3.8	
μ_2	TAPS	−	1.4 ± 0.2	3.3
		+	4.6 ± 2.1	
μ_2	EKC	−	0.9 ± 0.1	5.0
		+	4.5 ± 0.1	
μ_2	MR2034	−	1.1 ± 0.1	3.6
		+	4.0 ± 0.6	
μ_2	Naloxone	−	3.4 ± 0.2	0.9
		+	3.0 ± 0.4	

Table 3
Agonist activities for DAMGO and TAPS in the guinea pig ileum ($n = 4$–5). The concentrations producing 20% inhibition of contraction are indicated as IC_{20} (nM) values and were calculated from dose–response curves for the agonist to inhibit electrically stimulated contractions of isolated guinea pig ileum. The ileal preparations were treated with vehicle, the μ_1-opioid receptor antagonist naloxonazine (50 nM, washed before addition of agonist or nor-binaltorphimine) or naloxonazine and the κ-opioid receptor antagonist nor-binaltorphimine (20 nM)

Agonist	Control	Naloxonazine	Naloxonazine + nor-binaltorphimine
DAMGO	53	220	210
TAPS	21	190	470

responses, and then tested or were further treated with the κ_1-opioid receptor antagonist nor-binaltorphimine (20 nM), followed by opioid agonist treatment. Naloxonazine reduced the effects of TAPS and DAMGO in a similar manner. Addition of nor-binaltorphimine resulted in a further, albeit less pronounced shift of the dose–response curve for TAPS to the right (Fig. 2B).

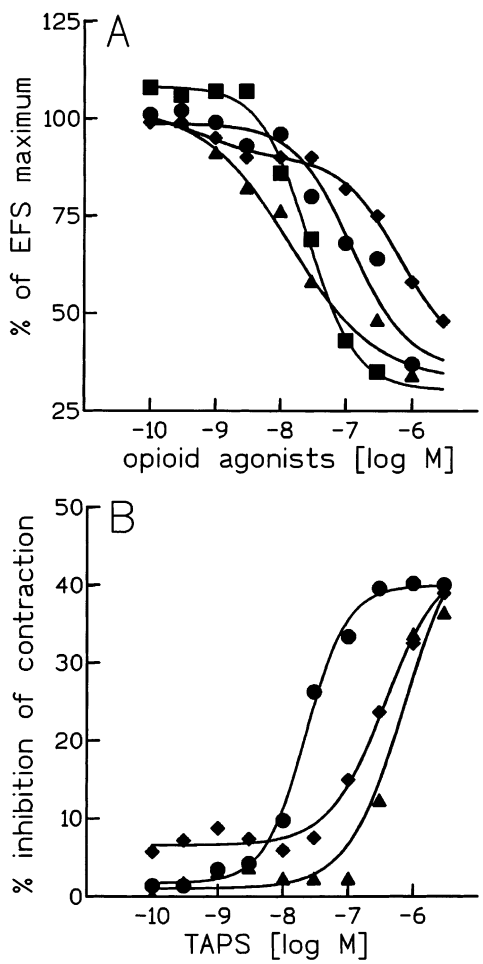

Fig. 2. (Panel A) Inhibition of guinea pig ileum electrically forced (EFS) contractions by various opioid agonists (filled triangles: TAPS, $n = 7$; filled squares: DAMGO, $n = 5$; filled circles: Dermorphin, $n = 5$; filled diamonds: DSTLE, $n = 3$). (Panel B) Antagonism of TAPS-induced inhibition (filled circles: controls, $n = 8$) of electrically stimulated guinea pig ileum contractions by naloxonazine (filled diamonds, $n = 4$) and naloxonazine plus nor-binaltorphimine (filled triangles, $n = 4$).

3.3. Effect of i.t. TAPS on antinociception and catalepsy

TAPS, when given i.t., produced a dose-dependent antinociceptive effect, which lasted more than 180 min following the administration of 100 pmol (Fig. 3A). The ED_{50} value for TAPS-induced antinociception was 18 pmol (range 14–23 pmol) (Fig. 3B), thus being less potent when compared to i.c.v. administration (Paakkari et al.,

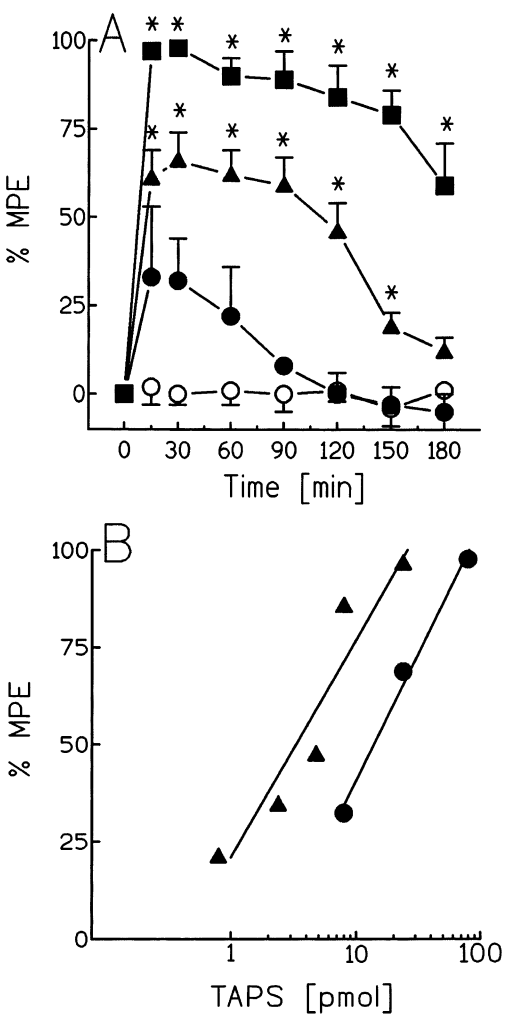

Fig. 3. (Panel A) Time–dose response for antinociception following i.t. administration of TAPS in rats (open circles: saline controls; filled circles: TAPS 10 pmol; filled triangles: TAPS 30 pmol; filled squares: TAPS 100 pmol). Data are presented as percentage of the maximum possible effect (% MPE) and represent means of six to eight separate experiments. $^{*}P < 0.05$ by Tukey's test. (Panel B) Dose response for antinociception after TAPS administration i.t. (filled circles) and i.c.v. (filled triangles). Data for i.c.v. injection are derived from Paakkari et al. (1993) for comparison.

1993). Naloxone completely abolished the TAPS-induced antinociception following i.t. injection (data not shown). Intravenous pretreatment with naloxonazine dose-dependently reduced the antinociceptive action of i.t. injected TAPS indicating a substantial involvement of the putative μ_1-opioid-receptor subtype in the generation of analgesia even on the spinal level (Fig. 4A, B). Similarly, i.t. application of naloxonazine resulted in an effective reduction of the i.t. TAPS effect (Fig. 5). Pretreatment with the δ-opioid receptor-selective antagonist naltrindole by itself did not produce an antinociceptive response. Further, naltrindole was unable to antagonize the effect of TAPS i.t. on antinociception (data not shown). The κ-opioid receptor antagonist nor-binaltorphimine blocked the TAPS effect only following pretreatment with a high, unselective dose

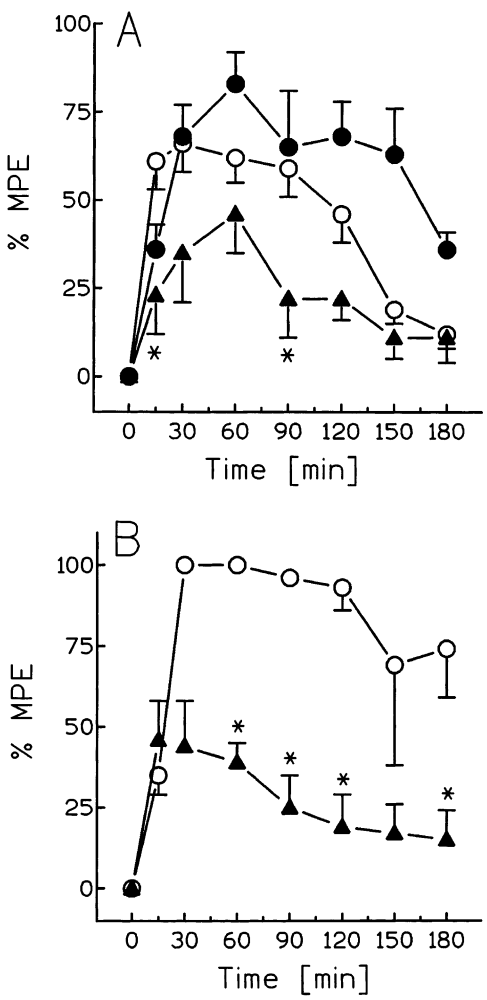

Fig. 4. Effect of the μ_1-opioid receptor antagonist naloxonazine on i.t. TAPS-induced antinociception. (Panel A) TAPS, 30 pmol, was injected i.t. in vehicle-treated (open circles, $n = 6$), and i.v. naloxonazine-pretreated (10 mg/kg, $n = 5$, filled circles; 20 mg/kg, $n = 7$, filled triangles) rats. (Panel B) TAPS, 100 pmol, was given i.t. in vehicle-treated (open circles, $n = 6$) and i.v. naloxonazine-pretreated (20 mg/kg) animals (filled triangles, $n = 5$). $^{*}P < 0.05$ (Tukey's test). Data are presented as percentage of the maximum possible effect (% MPE).

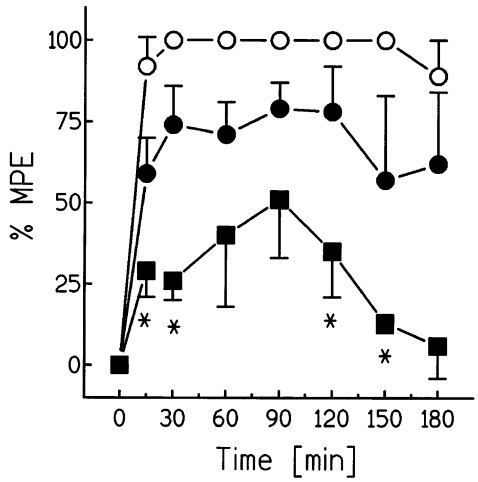

Fig. 5. Antagonism of i.t. TAPS (100 pmol)-induced spinal antinociception following i.t. pretreatment with naloxonazine. Naloxonazine dose-dependently antagonized the TAPS-induced antinociception following i.t. administration of 1 nmol (filled circles, $n = 4$) and 10 nmol (filled squares, $n = 4$). Open circles = TAPS without naloxonazine pretreatment. $^{*}P < 0.05$ (Tukey's test). Data are presented as percentage of the maximum possible effect (% MPE).

indicating a predominant non-κ-opioid receptor-related mode of action (data not shown).

In contrast to the i.c.v. route of treatment (Paakkari et al., 1993), TAPS i.t. did not produce catalepsy. The righting reflex was preserved in all animals independent of the dose of TAPS.

3.4. Effects of i.t. TAPS on ventilation

Following the i.t. injection of TAPS, there was a decrease in respiration rate, accompanied by an increase in tidal volume, resulting in a slight increase of the ventila-

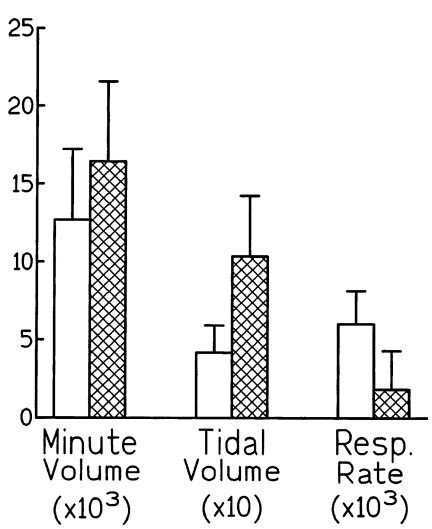

Fig. 6. Effect of TAPS following i.t. administration on respiratory minute volume, tidal volume, and respiration (Resp.) rate. The bars (open bars = vehicle, $n = 4$; crosshatched bars = TAPS, 100 pmol i.t., $n = 5$) represent integrated curve areas following i.t. treatments as described.

tion minute volume. However, statistical analysis (repeated measures ANOVA) did not reveal significant differences between the treatment and control groups (data not shown) over the observation period of 3 h. Correspondingly, the calculated curve areas were similar to controls (Fig. 6).

4. Discussion

The present study demonstrated that the dermorphin-derived tetrapeptide, Tyr-D-Arg^2-Phe-Sar^4 (TAPS) exhibits high selectivity to μ-opioid receptors. The K_i for TAPS displacement of [^{3}H]DAMGO from bovine thalamic membranes was over 1000-fold less than its K_i for displacement of the delta ligand [^{3}H]DPDPE. TAPS did not bind to κ_1-opioid receptor subtype binding sites ($K_1 > 1$ μM). However, in the κ_2-opioid receptor subtype selective assay, TAPS showed some, albeit low, affinity to these binding sites in a micromolar range. In a previous study, the μ/δ-selectivity ratio of a dimeric TAPS peptide was found to be 187 when binding studies were done on rat brain synaptosomes using [^{3}H]DAMGO and [^{3}H]DADLE in the presence of morphiceptin as μ- and δ-opioid receptor-selective assays, respectively (Lazarus et al., 1989). In our study, the μ/δ-selectivity ratio of TAPS using [^{3}H]DPDPE for δ-opioid receptor-specific binding was over 1000, possibly due to the superior selectivity of DPDPE to δ-opioid receptors as compared to the combination of [^{3}H]DADLE and the μ-opioid receptor agonist morphiceptin utilized by Lazarus et al. (1989).

Our previous studies have suggested that the in vivo activity of TAPS is consistent with selectivity at the proposed μ_1-opioid receptor subtype. Thus, TAPS produced potent antinociception and catalepsy, which were antagonized by naloxonazine, a μ_1-opioid receptor subtype-selective noncompetitive opioid antagonist (Paakkari et al., 1993). In striking contrast to morphine or dermorphin which are strong agonists at both μ_1- and μ_2-opioid receptor sites and depress respiration via a μ_2-opioid receptor-mediated mechanism (Ling et al., 1985; Paakkari et al., 1989, 1993), TAPS at equianalgetic doses did not produce respiratory depression but elicited stimulation of the respiration rate and ventilation minute volume which was blocked by naloxonazine (Paakkari et al., 1993). A potential antagonist action at μ_2-opioid receptor binding sites was first suggested by the same authors showing that i.c.v. pretreatment with TAPS of rats which also had received naloxonazine to selectively block μ_1-opioid receptor binding sites lead to a reversal of dermorphin-induced respiratory depression, a proposed μ_2-opioid receptor subtype-related effect. Further, TAPS was shown to antagonize the fentanyl-induced reduction of cAMP formation in human SH-SY5Y neuroblastoma cells, which appears to be mediated via μ_2-opioid receptors, corroborating an antagonist property of TAPS at this opioid-binding site (Smart and Lambert, 1996).

Since the binding studies revealed high affinity binding of TAPS at both μ-opioid receptor subtypes, we explored its putative differential agonist properties. In the first set of studies, binding of TAPS in the μ-opioid receptor-selective assays was repeated in the presence of sodium chloride and the stable GTP analog Gpp(NH)p. This treatment results in the uncoupling of the receptor molecule from the G-protein complex, leading to a drastic reduction of agonist binding at μ-opioid receptors without significantly affecting antagonist binding (Childers and Snyder, 1978; Puttfarcken et al., 1988; Clark et al., 1988; Werling et al., 1988). In the presence of sodium and guanosine nucleotides, the affinity of TAPS at the μ_1-opioid receptor subtype was reduced significantly, indicating that TAPS is a full agonist at these sites. Inclusion of sodium and Gpp(NH)p into the μ_2-opioid receptor-selective assay lead to a moderate reduction of TAPS affinity, which was less than its affinity change ("G-shift") in the μ_1-opioid receptor-selective assay and within the range of the G-shifts obtained for compounds such as EKC and MR2034, two κ-opioid receptor agonists with μ_2-opioid receptor antagonist properties (Wood et al., 1982). This finding may indicate that TAPS acts as a full agonist at the proposed μ_1-opioid receptor subtype but exhibits partial antagonist activity at μ_2-opioid binding sites. The binding of the full antagonist at both sites, naloxone, was not affected by sodium and Gpp(NH)p. This finding would be in accordance with the selective μ_1-opioid receptor agonist activity of TAPS in the in vivo studies (Paakkari et al., 1989, 1993). Technical problems, however, may restrict the use of G-shifts in our study. As the radioligands used for both μ_1- and μ_2-opioid receptor-selective assays are agonists, addition of sodium and Gpp(NH)p results in a reduction of specific binding of the radioligand itself. Whether this may affect the affinities of competing ligands remains unclear. However, the lack of effect on naloxone binding indicates that the measured affinities for the remainder of the tested compounds represent the proper effect of sodium and Gpp(NH)p on the competing ligands.

The functional data collected from the electrically stimulated guinea pig ileum indicated that TAPS was slightly more potent to inhibit contractions compared to DAMGO. Pretreatment with naloxonazine resulted in a 9-fold reduction of potency as compared to a 4-fold reduction when DAMGO was used as agonist. Thus, both TAPS and DAMGO appear to act on guinea pig ileum myenteric plexus neurons via μ_1-opioid receptor binding sites, while morphine appears to prefer μ_2-opioid sites to inhibit ileal contractions. The latter was shown by Gintzler and Pasternak (1983), who found that the initial inhibition of the morphine-induced reduction of electrically stimulated contractions of ileal segments after addition of naloxonazine was reversible after several washes. Since μ_1-opioid receptor binding site occupation of naloxonazine is believed to be non-competitive and irreversible in nature—the opposite being held true for its actions at the μ_2-opioid receptor

binding site—a predominant μ_2-opioid binding site-related mode of action for morphine was proposed (Gintzler and Pasternak, 1983).

Inhibition of κ-opioid receptors using nor-binaltorphimine further antagonized the TAPS-but not the DAMGO-induced inhibition of ileal contractions (Table 3). The mechanism of this action remains unclear. Nor-binaltorphimine is considered a κ-opioid receptor antagonist with selectivity towards the κ_1-opioid receptor subtype (Butelman et al., 1993; Nock et al., 1990). Concerning the receptor affinity of nor-binaltorphimine to κ_1- and κ_2-opioid receptor subtypes conflicting reports exist. Using [^{3}H]EKC to label κ_2-opioid receptors, nor-binaltorphimine showed more than 1000-fold selectivity for the [^{3}H]U69593-labeled κ_1-opioid receptor binding site (Nock et al., 1990). Recently, [^{3}H]nor-binaltorphimine was shown to bind to both κ_1- and κ_2-opioid receptor subtypes with affinities of 0.2 and 17.1 nM, respectively (Márki et al., 2000). On the other hand, because of the complete lack of affinity of TAPS to κ_1-opioid receptor binding sites, only κ_2-opioid receptor binding sites, where TAPS exhibits at least some affinity (Table 1), may account for the interaction of TAPS with nor-binaltorphimine in the guinea pig ileum. Considering the selectivity of nor-binaltorphimine for the κ_1-opioid receptor subtype (Nock et al., 1990; Márki et al., 2000) and the low affinity of TAPS at the κ_2-site, however, it appears unlikely that the TAPS-induced effects on the guinea pig ileum would be mediated through κ-opioid receptors. Since nor-binaltorphimine exhibits some, albeit low, affinity to μ-opioid receptors compared to κ_1-opioid receptors, its effect in the guinea pig ileum may be due to an unspecific interaction at μ-opioid receptor sites, especially at the rather high concentration of 20 nM which was used in these experiments. Thus, the K_i for nor-binaltorphimine at μ-opioid receptors using [^{3}H]DAMGO was shown to range from 8 (Emmerson et al., 1994) to 42 nM (Nock et al., 1990). Moreover, DAMGO has been shown to bind to the κ_2-opioid receptor subtype with a similar K_i as TAPS when [^{3}H]nor-binaltorphimine was used as radioligand (Márki et al., 2000). Therefore, it would be expected that the inclusion of nor-binaltorphimine in the guinea pig ileum assay using DAMGO as agonist would render a similar augmentation of the antagonist action of naloxonazine as in the experiments using TAPS as agonist. Due to the lack of such an effect (Table 3), however, it appears unlikely that the augmented antagonism of TAPS action after addition of nor-binaltorphimine would be due to displacement of TAPS from μ-opioid receptor binding sites.

TAPS exerts potent antinociceptive properties when administered either i.c.v., i.v., p.o. (Paakkari et al., 1993) and s.c. (Sasaki et al., 1984) to rats. The present data demonstrate that this enhanced action compared to morphine and enkephalins and their derivatives is due to an increased affinity to μ-opioid receptors. While the affinities of the parent peptide dermorphin in the μ_1- and μ_2-opioid receptor subtype-specific assays in our hands are similar to TAPS (0.4 and 0.9 nM, respectively), the duration of the antinociceptive effect of TAPS is prolonged compared to dermorphin. Thus, following i.c.v. administration of 10–11 pmol (Paakkari et al., 1993; Sato et al., 1987) and of 100 pmol i.t., the resulting antinociceptive effect lasted more than 3 h. This phenomenon probably results from an increased enzymatic stability due to the D-Arg^2- and the synthetic Sar^4-amino acid residues of the tetrapeptide molecule (Sato et al., 1987).

TAPS i.t. produced a dose-dependent antinociceptive effect in the tail-flick latency test. The potency was about 5-fold less, when compared to the i.c.v. administration. Naloxonazine dose-dependently reduced the antinociceptive effect when given i.v. and i.t. 24 h prior to i.t. injection of TAPS, indicating that naloxonazine-sensitive μ_1-opioid receptor binding sites are not only involved in the supraspinal but also in the spinal antinociception produced by TAPS i.t.

In contrast to the i.c.v. route of administration (Paakkari et al., 1993), i.t. application of TAPS did not produce any significant changes in respiratory patterns. Thus, retrograde flow of i.t. infused TAPS peptide in a cranial direction towards the medulla does not appear to be substantial, emphasizing, together with the lack of catalepsy, the true spinal origin of the antinociceptive properties. This was also confirmed macroscopically through injection of methylene blue shortly before the animals were sacrificed.

The definite proof for the existence of μ-opioid receptor subtypes remains elusive to date. While three distinct genes were identified, encoding separate proteins identified as the δ-(OP_1), κ-(OP_2), and μ-(OP_3) opioid receptors (Dhawan et al., 1996), no other genes could be identified encoding the otherwise pharmacologically identifiable receptor subtypes. Due to the presence of introns, however, receptor heterogeneity may be possible via alternate splicing. Indeed splice variants have been identified recently for the μ-opioid receptor (Pan et al., 1999, 2000), which exhibit subtle differences in their affinities to μ-opioid receptor agonists and appear to be differentially distributed in the brain (Pan et al., 1999). In studies using cell lines transfected to express different splice variants of the μ-opioid receptor, TAPS may be instrumental in order to conclusively elucidate the structure of the proposed μ_1- and μ_2-opioid receptor subtypes by means of its agonist vs. antagonist action.

TAPS exerts potent antinociceptive effects following various routes of administration including p.o. (Paakkari et al., 1993; Table 4), both, at spinal and supraspinal levels. Its efficacy at supraspinal sites is about 5-fold higher compared to spinal sites. In the present study, naloxonazine dose dependently reduced the effect of i.t. TAPS on spinal antinociception to a major part, indicating a predominant naloxonazine-sensitive, μ_1-opioid receptor subtype-related action and possibly a minor naloxonazine-insensitive component, presumably via μ_2-opioid receptor bind-

Table 4
Comparison of the pharmacological properties of TAPS following i.c.v. (Paakkari et al., 1993) and i.t. administration

Effect	i.c.v.	i.t.
Analgesia ED_{50}	4 pmol	18 pmol
Catalepsy	yes	no
Respiration	stimulation	no effect
Heart	tachycardia	not tested
Proposed mechanism	μ_1-opioid receptor site	μ_1-opioid receptor site

ing sites. Similarly, the antinociceptive effect of TAPS following i.c.v. administration was antagonized after pretreatment with naloxonazine. Catalepsy after TAPS i.c.v. administration is induced through supraspinal mechanisms (Paakkari et al., 1993), whereas its intrathecal administration was devoid of a cataleptic response. The supraspinal cataleptic effect was blocked by naloxonazine, again indicating the possible involvement of μ_1-opioid receptor binding sites. Further, TAPS acts as a respiratory stimulant at supraspinal μ-opioid receptors. This effect was equally blocked by naloxonazine. i.t. administration of TAPS did not produce any effects on respiratory parameters. Following i.c.v. administration in rats, TAPS elicited an increase of heart rate, while the parent peptide dermorphin was less active (Paakkari et al., 1992). Only after blocking the proposed μ_1-opioid receptor subtype with naloxonazine, TAPS like dermorphin produced a decrease of heart rate, presumably due to activity at the μ_2-opioid receptor subtype. Correspondingly in the present receptor binding studies, TAPS exhibits high affinity to naloxonazine-sensitive, μ_1-opioid receptor binding sites which is about 3-fold higher than its affinity at the proposed μ_2-opioid receptor binding site.

The selectivity of TAPS for the μ_1-opioid receptor binding site, combined with a possible antagonist action at the proposed μ_2-opioid receptor binding site may render this peptide as a candidate lead compound for the development of μ_1-opioid receptor-selective agents. Moreover, this synthetic peptide may prove clinically useful as a potent analgesic drug, lacking the feared respiratory depressive side effects in the management of acute and chronic pain.

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