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Characterization of binding kinetics of [^{3}H]Tyr-D-Arg^2-Phe-Sar^4 at opioid receptors

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Abstract

The dermorphin-derived, MOP receptor-selective tetrapeptide Tyr-D-Arg^2-Phe-Sar^4 (TAPS) exhibits a high antinociceptive potency and stimulates respiration in rats. The receptor binding kinetics of [^{3}H]TAPS were investigated using crude calf thalamic membrane preparations. Saturation studies showed binding of [^{3}H]TAPS at two binding sites (0.4 and 3.2 nM). In the presence of $MgSO_4$, [^{3}H]TAPS binding occurred with high affinity at a single site only. The high-affinity binding component was reduced following the addition of K_2-EDTA. The increase of the apparent dissociation constant was due to an enhanced dissociation rate ($P<0.05$), while association rates remained unchanged. Addition of 5′-guanylylimidodiphosphate (Gpp(NH)p) resulted in a reduction of affinity which was augmented in the presence of Na^+. Thus, [^{3}H]TAPS, depending on the presence of divalent cations, serves as ligand at two MOP receptor binding sites in the calf thalamus, which may represent distinct affinity states of the same receptor or receptor subtypes thereof.

Keywords: Dermorphin; Tetrapeptide; TAPS; Receptor binding; Kinetic; Brain

1. Introduction

Dermorphin, a heptapeptide extracted from the skin of *Phyllomedusa sauvagei* (Montecucchi et al., 1981), is a potent MOP (formerly termed μ-opioid) receptor agonist which exerts an antinociceptive potency exceeding that of morphine by 752 to 2170 times (Broccardo et al., 1981; Stevens and Yaksh, 1986). It represents a unique, naturally occurring peptide due to the presence of a D-amino acid residue (D-Ala^2). This residue appears essential for the binding of dermorphin to MOP receptors (Giagnoni et al., 1987; Guglietta et al., 1987). The N-terminal tetrapeptide (*H*-Tyr-D-Ala-Phe-Gly) of dermorphin was shown to be the minimum sequence required to produce an antinociceptive, albeit reduced effect (Broccardo et al., 1981). The efficacy of the N-terminal tetrapeptide is further enhanced by the substitution of the D-Ala^2 residue with D-Arg and Gly^4 with the synthetic amino acid analog sarcosine (Sar) (Sato et al., 1987). The resulting tetrapeptide Tyr-D-Arg^2-Phe-Sar^4 (TAPS) is three times more potent than dermorphin in antinociceptive tests in rats (Sato et al., 1987). It is an effective antinociceptive compound both at the supraspinal (Paakkari et al., 1993) and spinal (Vonhof et al., 2001) level. While a mechanism of action mediated by other opioid receptors cannot entirely be ruled out, the potent activity of small concentrations of TAPS in a picomolar range together with its high selectivity at the MOP receptor in receptor binding studies (Vonhof et al., 2001) and the complete reversal of its pharmacological actions by naloxone and naloxonazine (Paakkari et al., 1993) render actions of this peptide at binding sites other than the MOP receptor extremely unlikely. Strikingly, TAPS was shown in rats to elicit respiratory stimulation, which was blocked by naloxonazine indicating an agonist action at the putative MOP_1 receptor binding site (Paakkari et al., 1993) according to the dual MOP-subtype hypothesis proposed by Goodman and Pasternak (1985). In contrast, naloxonazine pretreatment caused an augmentation of the ventilatory depressant action of the parent peptide dermorphin (Paakkari et al., 1990).

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A partial agonist/antagonist action for TAPS at the proposed MOP_2 receptor binding site was suggested by evidence, showing that the combined pretreatment of the animals with naloxonazine and TAPS effectively blocked the ventilatory depressant action of dermorphin (Paakkari et al., 1993). Correspondingly, receptor binding studies using established subtype-specific receptor binding assays supported a potential partial agonist/antagonist mode of action at the MOP_2 receptor binding site (Vonhof et al., 2001). Furthermore, TAPS antagonized the fentanyl-induced reduction of cAMP formation in human SH-SY5Y neuroblastoma cells, a putative MOP_2 receptor-mediated effect (Smart and Lambert, 1996). The receptor binding kinetics of TAPS have not been fully characterized so far. Since the opioid receptor-specific assay systems utilize radioligands which are not necessarily specific for the subtype under investigation, blocking concentrations of other, partly selective compounds are needed in order to reduce binding at other sites. TAPS as competitor in these assays may interfere with these other sites, thus potentially reducing the subtype-specificity of the assay systems. We therefore extended the characterization of TAPS binding to opioid receptors using tritiated TAPS as radioligand.

2. Materials and methods

Calf thalamic tissue has been extensively used to characterize MOP receptor subtype-specific binding. For comparability reasons and in order to characterize binding of [^{3}H]TAPS to putative MOP receptor subtypes, crude membrane preparations of calf thalamus were prepared as described in the literature (Clark et al., 1988). Briefly, calf medial thalami, obtained freshly from a local slaughterhouse and processed immediately, were homogenized in 35 volumes of Tris–HCl buffer using a Brinkmann Polytron homogenizer and centrifuged at 49 000 × g and 4 to 6 °C for 40 min. The supernatants were discharged and the pellets were 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 50 mM Tris–HCl buffer or potassium phosphate buffer at 15 mg tissue wet weight/ml (ww/ml) and a total incubation volume of 3 ml. Preliminary experiments revealed a slight, approximately 10% improvement of specific binding capacity for Tris–HCl buffer compared to potassium phosphate buffer. Binding of [^{3}H]TAPS was performed at 25 °C over a period of 5 h.

Saturation, competition and association/dissociation experiments were performed as described in the literature (Bylund and Yamamura, 1990). For saturation experiments, the tissue resuspensions were incubated with increasing concentrations of labeled TAPS. Competition experiments were performed using a constant concentration of radioligand ranging around its previously determined dissociation constant (0.5 to 0.7 nM). For association studies, [^{3}H]TAPS (0.5 to 0.7 nM) was added to the tissue resuspension at various time points resulting in a range of incubation periods. Nonspecific binding was determined in the presence of 1 μM levallorphan. This concentration was chosen in accordance to the studies by Clark et al. (1988) in order to maintain comparable assay conditions. Levallorphan at this high concentration may not selectively bind at MOP receptors. However, given the high selectivity of TAPS binding at the MOP receptor at the low nanomolar radioligand concentrations used in the present experiments, displacement of [^{3}H]TAPS from sites other than MOP receptors appears unlikely. In dissociation experiments, 1 μM levallorphan was added following a 5-h pre-incubation with [^{3}H]TAPS (0.5 to 0.7 nM) in order to reach equilibrium conditions, and the incubation was then continued for various lengths of time. Saturation, association and dissociation experiments were performed in the absence or presence of 5 mM $MgSO_4$ or 1 mM K_2-EDTA. For the determination of the effect of G-protein coupling to the opioid receptor on [^{3}H]TAPS binding, 10 μM 5′-guanylylimidodiphosphate (Gpp(NH)p) was added to the incubation mixture in the presence or absence of 100 mM NaCl.

In all experiments, the incubation was terminated by vacuum filtration through Whatman GF/B glass filters using a 48-well cell harvester (Brandel, Gaithersburg, MD, USA), followed by three rinses of ice-cold Tris–HCl buffer. The filters were placed in glass scintillation vials, and 5 ml of water-miscible scintillation fluid (Beckman Ready-Solv HP) was added. Bound radioactivity was measured on the following day using a liquid scintillation counter (LKB 1218 Rackbeta). Dissociation (K_d) and inhibition (K_i) constants were determined following Scatchard or Hofstee transformation, respectively, using the radioligand-binding analysis software LIGAND (Elsevier Biosoft) (Munson and Rodbard, 1980). Statistical analysis of single vs. multiple binding site hypotheses was performed using the F-test as described (Munson and Rodbard, 1980). The kinetic data from the association experiments were transformed assuming pseudo-first-order conditions as described by Weiland and Molinoff (1981). Apparent association constants (k_{obs}) were derived from the slope of a plot of $\ln[B_e/(B_e - B)]$ vs. time, where B represents specific binding of [^{3}H]TAPS at a given time point during the association experiment and B_e describes the amount of radioligand specifically bound at equilibrium. The association rate constants (k_{+1}) were calculated according to $(k_{obs} - k_{-1})/L_T$, with L_T representing the total concentration of radioligand. In general, specific binding was less than 5% of the added radioligand. Thus, reductions of free radioligand were neglected and its concentration at each time point T was assumed to remain constant. Dissociation rate constants (k_{-1}) were estimated from the slope of the straight line resulting from plotting $\ln(B/B_0)$ vs. time, where B_0 represents total specific binding before addition of levallorphan (Weiland and Molinoff, 1981).

Unless stated otherwise, figures and tables represent mean values ± S.E.M. of specific binding derived from at

least three separate experiments with data points determined in duplicates. Unlabeled TAPS, Tyr-D-Ala-Gly-(Me)Phe-Gly-ol (DAMGO), [D-Ala^2, D-Leu^5]enkephalin (DADLE), [D-Ser^2, Leu^5]enkephalin (DSTLE), *H*-D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2 (CTOP), [D-Pen^2, D-Pen^5]enkephalin (DPDPE), (5α,7α,8β)-(+)-*N*-methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspirol[4,5]dec-8-y-]-benzeneacetamide (U69593), *trans*-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl) cyclohexyl] benzene acetamide (U50488) and dermorphin were obtained from Peninsula (Belmont, CA). Naloxone was purchased from DuPont Pharmaceuticals, Natick, MA. Magnesium-, manganese-, Na^+ salts and Gpp(NH)p were purchased from Sigma (St. Louis, MO). TAPS was custom-tritiated (Amersham, Arlington Heights, IL) resulting in a specific activity of 1.75 TBq/mmol. For the calculation of various binding parameters, the specific activity was adjusted for the elapsed radioactive decay in all binding experiments. Statistical evaluation of differences of rate constants was performed using the Kruskal–Wallis test followed by the Mann–Whitney *U*-test.

3. Results

3.1. Effect of pH and incubation temperature on [^{3}H]TAPS binding

Specific [^{3}H]TAPS binding was maximal within a range of pH 7.0 to 7.6. Tris–HCl proved slightly superior to potassium phosphate buffer regarding maximum binding. The amount of bound radioligand was low at temperatures below 10 °C. Maximum binding was achieved at 25 °C, followed by a drastic reduction at higher incubation temperatures. Specific binding was linear up to a tissue concentration of 20 mg ww/ml and a total incubation volume of 4 ml. The lowest levels of nonspecific binding of [^{3}H]TAPS were measured between 15 and 20 mg ww/ml and total incubation volumes of 2 to 4 ml. Nonspecific binding was linear within this range of tissue concentrations, and incubation volumes and specific binding amounted to >90% of the total binding. For the remaining experiments, bovine

Table 1
Effect of cations on [^{3}H]TAPS binding

	Concentration (mM)	[^{3}H]TAPS binding (%)
$MgSO_4$	5	+54.6 ± 11.0
$MgCl_2$	5	+52.4 ± 12.9
$MnCl_2$	5	+81.8 ± 4.9
$CaCl_2$	5	+13.5 ± 10.2
NaCl	5	−1.2 ± 1.8
NaCl	100	−70.8 ± 1.2
KCl	5	+4.0 ± 2.1

Percent changes of specific binding are reported as means of three to four separate experiments ± S.E.M. The amount of specific binding without added cations in control experiments under similar incubation conditions was set at 100%.

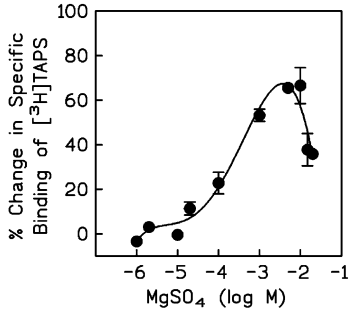

Fig. 1. Change of specific [^{3}H]TAPS binding to thalamic membranes with increasing concentrations of $MgSO_4$. Crude calf thalamic membranes were incubated in the presence of increasing concentrations of $MgSO_4$, using [^{3}H]TAPS for 5 h at 25 °C. The data points represent means ± S.E.M. for three separate experiments.

thalamic membranes were incubated with [^{3}H]TAPS at pH 7.5 in 50 mM Tris–HCl buffer in a total volume of 3 ml at 15 mg ww/ml concentration in order to maintain optimal assay conditions. Due to the inhibitory effect of TAPS on enkephalin-degrading enzymes and its enhanced stability against degradation (Sato et al., 1987), TAPS retains its activity under these conditions for a prolonged time.

3.2. Effect of cations on [^{3}H]TAPS binding

Bivalent cations were shown to enhance specific binding of a variety of MOP receptor ligands (Kinouchi et al., 1990). The inclusion of cations enhanced specific binding of [^{3}H]TAPS to thalamic membranes (Table 1). Among the cations tested, $MnCl_2$ was most effective in enhancing binding of [^{3}H]TAPS, resulting in an 81.8% increase of specific binding, followed by $MgSO_4$ and $MgCl_2$. As for $MgSO_4$, maximum enhancement of specific [^{3}H]TAPS binding was shown between 5 and 10 mM followed by a decline at higher concentrations (Fig. 1). NaCl concentration-dependently reduced specific binding (Table 1). Inclusion of K_2-EDTA in the assay in order to remove soluble cations resulted

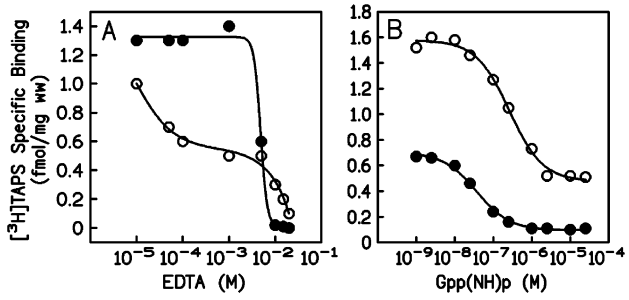

Fig. 2. Effect of K_2-EDTA and Gpp(NH)p on specific [^{3}H]TAPS binding to crude calf thalamic membranes. Panel A: K_2-EDTA was added in increasing concentrations to the incubation mixture (open circles, $n = 3$). Filled circles depict the effect of co-incubation of 5 mM $MgSO_4$. Panel B: specific [^{3}H]TAPS binding was measured in the presence of increasing concentrations of Gpp(NH)p in the presence (filled circles) and absence (open circles) of 100 mM NaCl. The data points represent mean values of three separate experiments.

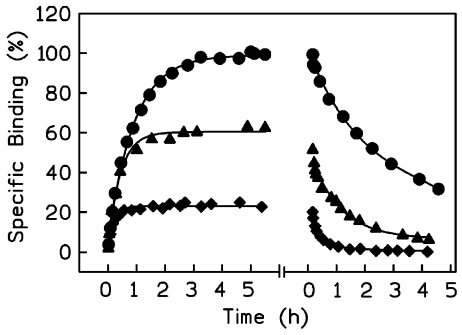

Fig. 3. Association and dissociation of [^{3}H]TAPS (0.5 to 0.7 nM) in the presence of 5 mM $MgSO_4$ (filled circles), 1 mM K_2-EDTA (filled diamonds) or in the absence of both (filled triangles). The level of specific [^{3}H]TAPS binding after a 5-h incubation time in the association experiments including 5 mM $MgSO_4$ at equilibrium was set at 100% (i.e. 7155 dpm) and served as the reference for the remainder of the data points. The left side of the figure represents the association experiments; the right side shows the results of the dissociation experiments. Dissociation of [^{3}H]TAPS was initiated following a 5-h incubation of thalamic membranes at 25 °C by addition of 1 μM levallorphan at time point "0". The data points represent mean values of three separate experiments.

in a biphasic reduction of specific binding (Fig. 2A). In these experiments, specific binding was restored after the addition of 5 mM $MgSO_4$. Since $MgSO_4$ has already been routinely utilized in MOP receptor binding assays with radioactive ligands such as [^{3}H]DAMGO (Clark et al., 1988), we included $MgSO_4$ unless stated otherwise in all subsequent assays for reasons of comparability.

3.3. Association and dissociation kinetics

Radioligand association and time to reach equilibrium conditions were dependent on the presence of Mg^{2+} cations. Thus, equilibrium conditions were reached after 3 h of incubation when 5 mM Mg^{2+} was present in comparison to 2 h (without Mg^{2+} added) and 0.5 to 1 h in the presence of 1 mM K_2-EDTA (Fig. 3). The association rate constants were similar under these conditions (P = n.s., Table 2). In the dissociation experiments, the rate of dissociation of the radioligand was dependent on the presence of Mg^{2+}. When cations were removed using K_2-EDTA, virtually the entire specifically bound [^{3}H]TAPS was dissociated from the receptor within 1 h. Correspondingly, the dissociation rate constant was highest, when K_2-EDTA was present in the incubation mixture (Table 2). With the inclusion of 5 mM $MgSO_4$, dissociation of the radioligand was reduced (Fig. 3, Table 2). Thus, even when the radioligand was allowed to dissociate from the receptor for 4.5 h, about 40% of specific binding remained. The calculated dissociation constants (K_D) of 0.3 nM (+5 mM $MgSO_4$), 1.0 nM (without addition of $MgSO_4$ or K_2-EDTA) and 2.4 nM (+1 mM K_2-EDTA) were in close proximity to the apparent K_d's derived from saturation experiments shown below. The K_D was increased by almost 13-fold in the presence of 1 mM K_2-EDTA compared to the experiments with added $MgSO_4$.

Table 2
Association and dissociation rate kinetics of [^{3}H]TAPS

	Bound [^{3}H]TAPS (fmol/mg ww)	k_{-1} (min^{-1})	k_{+1} (min^{-1} nM^{-1})
+ $MgSO_4$ − K_2-EDTA	1.6 ± 0.0	0.006 ± 0.001	0.023 ± 0.001
− $MgSO_4$ − K_2-EDTA	0.9 ± 0.0	0.025 ± 0.003[a]	0.025 ± 0.010
− $MgSO_4$ + K_2-EDTA	0.4 ± 0.0	0.074 ± 0.008[a,b]	0.030 ± 0.007

The data are presented as means ± S.E.M. from three separate experiments. There were no statistical differences between the association rate constants.

[a] This denotes a statistically significant difference compared to the experiments including 5 mM $MgSO_4$ (P < 0.05).

[b] This signifies a statistical difference in comparison to the assays without K_2-EDTA or added $MgSO_4$ (P < 0.05).

Table 3
Saturation studies of [^{3}H]TAPS receptor binding

K_2-EDTA (1 mM)	$MgSO_4$ (5 mM)	Binding sites	K_d (nM)	B_{max} (fmol/mg ww)
+	−	2	(a) 0.4 ± 0.3	(a) 0.2 ± 0.2
			(b) 3.5 ± 1.1	(b) 2.0 ± 0.2
−	−	2	(a) 0.4 ± 0.1	(a) 0.9 ± 0.3
			(b) 3.2 ± 3.0	(b) 0.9 ± 0.3
−	+	1	0.3 ± 0.0	2.8 ± 0.1

The data are presented as means ± S.E.M. for three to six separate experiments.

3.4. Saturation studies using [^{3}H]TAPS

In experiments using no additional supplementation of $MgSO_4$, computerized fitting of the resulting Scatchard plots revealed a significant better fit of a two-sided model (P < 0.05), suggesting the presence of two binding sites with about 8-fold different apparent K_d's. The receptor densities for both binding sites were approximately equal (Table 3).

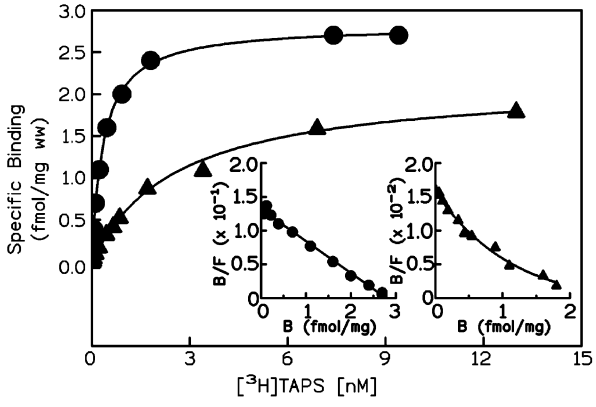

Fig. 4. Saturation binding isotherms for [^{3}H]TAPS using crude thalamic membrane preparations. The data points represent mean values for three to six separate experiments in the presence of 5 mM $MgSO_4$ (filled circles) or 1 mM K_2-EDTA (filled triangles). The insets show the resulting Scatchard plots for [^{3}H]TAPS saturation including $MgSO_4$ (left insert) or K_2-EDTA (right insert).

Table 4
Effect of Gpp(NH)p and Na^+ on [^{3}H]TAPS receptor binding

K_2-EDTA (1 mM)	$MgSO_4$ (5 mM)	Gpp(NH)p (10 μM)	NaCl (100 mM)	Binding sites	K_d (nM)	B_{max} (fmol/mg ww)
+	–	–	–	2	(a) 0.5 ± 0.3 (b) 3.4 ± 1.1	(a) 0.4 ± 0.4 (b) 2.4 ± 0.3
+	–	–	+	1	1.9 ± 0.9	0.3 ± 0.1
+	–	+	–	1	1.6 ± 0.1	1.5 ± 0.1
+	–	+	+	1	3.3 ± 0.8	0.6 ± 0.1
–	+	–	–	1	0.5 ± 0.0	2.7 ± 0.1
–	+	–	+	1	0.8 ± 0.1	1.9 ± 0.1
–	+	+	–	1	0.9 ± 0.1	1.9 ± 0.1
–	+	+	+	1	2.8 ± 0.7	1.8 ± 0.5

The data are presented as means ± S.E.M. of three to six separate experiments.

Addition of 1 mM K_2-EDTA resulted in a reduction of the overall specific binding mainly due to a reduction of the high-affinity binding site, whereas the amount of low affinity binding sites was increased. The curvilinear fit, indicating the presence of two sites with different affinity for TAPS, remained statistically superior to the single-site model ($P < 0.05$, Fig. 4). The apparent affinity of the radioligand at both binding sites remained unchanged. When 5 mM $MgSO_4$ was added to the incubation medium, however, the presence of a single high-affinity binding site was evident (Table 3, Fig. 4).

3.5. Modulation of specific binding of [^{3}H]TAPS by Gpp(NH)p and Na^+

The inclusion of a stable GTP analog, Gpp(NH)p, resulted in a dose-dependent reduction of specific [^{3}H]TAPS binding, which was further reduced in the presence of 100 mM NaCl (Fig. 2B). There was a reduction of affinity of the radioligand both in the presence or absence of K_2-EDTA or $MgSO_4$ to a similar extent. This reduction of affinity was accompanied by a decrease in receptor density (Table 4). While control experiments without Gpp(NH)p or NaCl suggested the existence of two binding sites in the presence of K_2-EDTA as mentioned above ($P < 0.05$), addition of Gpp(NH)p, NaCl or $MgSO_4$ resulted in binding characteristics which, following Scatchard transformation, favored the single-binding site model (Table 4).

3.6. Competition studies using [^{3}H]TAPS as radioligand and various MOP-, DOP- and KOP receptor-selective agents

While DOP (δ) and KOP (κ) receptor-selective ligands showed only low affinity to sites labeled by [^{3}H]TAPS, MOP receptor-selective compounds were able to effectively inhibit the binding of [^{3}H]TAPS. Most pronounced, dermorphin competed for [^{3}H]TAPS binding with high affinity, corroborating the MOP receptor selectivity of TAPS (Table 5). The inhibitory constants (K_i) were 0.2 ± 0.1 nM (dermorphin), 3.6 ± 0.4 nM (CTOP) and 0.1 ± 0.0 nM (naloxone). The K_i for the DOP receptor-selective ligand DPDPE was 139.3 ± 3.8 nM and for the KOP receptor-selective compounds, 516.5 ± 28.2 nM (U69593) and 228.3 ± 12.5 nM (U50488). When MOP receptor-specific agents were tested for their ability to inhibit [^{3}H]TAPS binding in the presence of K_2-EDTA, their inhibitory constants were equally decreased by about 6-fold with the exception of DSTLE which suffered a 16-fold reduction of its K_i (Table 5). Hill coefficients (n_H) were close to unity in all experiments.

4. Discussion

TAPS, a dermorphin-derived tetrapeptide, has received increasing attention because of its differential opiate-like properties in pharmacological studies. TAPS was shown, in contrast to morphine, to stimulate respiration in the face of an enhanced antinociceptive potency (Paakkari et al., 1993). TAPS may be instrumental in the elucidation of the cellular and molecular mechanisms mediating antinociception and respiratory depression. The mechanisms underlying these effects may not be necessarily identical and may follow along hypotheses involving subtypes of receptors. These receptor subtypes may mediate the single effects, desired or undesired, following opiate administration. On the other hand, the multiplicity of opiate effects on nociceptive, behavioral and vegetative systems may result from a widespread distribution of opioid receptors of the same type, i.e. of the MOP-, DOP- or KOP-type, on specialized cells in distinct regions of the central nervous system which are involved in the control of these systems.

Table 5
Competition of MOP receptor ligands for [^{3}H]TAPS binding

Competitor	+ $MgSO_4$ (5 mM)			+ K_2-EDTA		
	K_i (nM)	B_{max} (fmol/mg)	n_H	K_i (nM)	B_{max} (fmol/mg)	n_H
DAMGO	0.4 ± 0.1	2.7 ± 0.3	0.93 ± 0.04	2.4 ± 0.4	2.5 ± 0.3	1.14 ± 0.06
Morphine	0.8 ± 0.4	2.7 ± 0.0	0.81 ± 0.15	4.6 ± 0.3	3.2 ± 0.1	0.95 ± 0.05
DSTLE	2.7 ± 0.1	1.9 ± 0.1	0.96 ± 0.01	43.9 ± 11.6	2.5 ± 0.5	0.75 ± 0.02
DADLE	3.1 ± 0.4	2.1 ± 0.1	1.18 ± 0.06	18.3 ± 1.1	2.7 ± 0.3	0.86 ± 0.10

Radioligand concentration was maintained at 0.5 nM. Data represent means ± S.E.M. of three to four separate experiments (n_H = Hill coefficient).

In previous studies, we characterized the ability of TAPS to compete with radioligands at opioid receptors using various opioid receptor-subtype-selective binding assay systems (Vonhof et al., 2001). TAPS was shown to bind with high affinity to MOP receptor subtypes as proposed by Ling and Pasternak (1982) and Ling et al. (1985), using the MOP_1- and MOP_2-opioid receptor subtype-specific assay setups described by Clark et al. (1988). Receptor binding of TAPS was highly MOP receptor-selective, as shown by low affinities in DOP-, KOP_1- and KOP_2-opioid receptor-specific assays using [^{3}H]DPDPE, [^{3}H]U69593 and [^{3}H]Ethylketocyclazosine ([^{3}H]EKC), respectively (Clark et al., 1986; Zukin et al., 1988). The selectivity ratios compared to the affinity of TAPS at MOP receptors were 214 (KOP_2), 1124 (DOP) and >20 000 (KOP_1), indicating that at nanomolar concentrations, TAPS exclusively binds to MOP receptors. Unfortunately, definite proof for the existence of MOP receptor subtypes is still lacking, despite a sizable amount of evidence gathered from pharmacological experiments. So far, there is evidence for only one gene encoding the MOP receptor in rats (Chen et al., 1993) and humans (Wang et al., 1993). However, the existence of different splice variants of the transcribed mRNA, which translate into functional receptor proteins with subtle differences in affinity to MOP receptor agonists and a differential distribution in the brain, has been reported recently (Abbadie et al., 2000a,b; Pan et al., 1999, 2000). Additionally, treatment with antisense oligonucleotides inhibiting the expression of selected splice variants in mice resulted in differential changes of the antinociceptive response following morphine and morphine-6β-glucuronide treatment (Rossi et al., 1995; Schuller et al., 1999). Although these reports support the hypothesis of the existence of MOP receptor subtypes, it remains unclear whether and which of these variants represent the molecular correlates of the proposed MOP_1 and MOP_2 receptor subtypes as characterized in pharmacological studies.

Furthermore, the definition of TAPS binding at the proposed MOP receptor subtypes via subtype-specific assays is not entirely unequivocal since the receptor binding assays used to identify MOP_1- and MOP_2-opioid receptor subtypes may not be entirely specific. Thus, [^{3}H]DADLE, which is used as radioligand in the MOP_1 receptor-specific assay (Clark et al., 1988), also exerts high affinity at DOP receptors. In this assay, DPDPE must be co-incubated with [^{3}H]DADLE to block radioligand binding at the DOP receptor, resulting in an increasingly complex situation in the reaction tube, especially in the presence of additional competitors such as TAPS. The same complexity may pose a problem for the proposed MOP_2 receptor subtype-specific assay using [^{3}H]DAMGO and DSTLE to block radioligand binding at the MOP_1 binding site. Therefore, the use of [^{3}H]TAPS as radioligand appeared a prudent approach for further characterization of its receptor binding properties based on its strong MOP receptor selectivity.

The present saturation studies show binding of [^{3}H]TAPS to crude thalamic membranes at a low nanomolar range in the presence of $MgSO_4$. When radioligand saturation binding was performed without additional $MgSO_4$, [^{3}H]TAPS bound at an additional binding site with about 8-fold lower affinity. When free cations were removed from the incubation mixture through addition of K_2-EDTA, the presence of two distinct binding sites was still evident. The number of the high-affinity sites was reduced, however, to about 10% of the lower-affinity site. The ‘‘native’’ conditions, i.e. when neither K_2-EDTA nor $MgSO_4$ were added to the incubation mixture, resulted in a mixed receptor composition with each binding site accounting for about 50% of the total binding. This pattern may be due to endogenous cations such as Mg^{2+} or Mn^{2+} in the tissue preparation. It remains unclear whether the observed binding sites with different affinity for TAPS represent MOP_1 or MOP_2 receptor subtypes. Rather, binding may occur to the same receptor protein, which may exist in two affinity states. Thus, when the maximum densities (B_{max}) for both sites under EDTA conditions are added together, the total concentration of [^{3}H]TAPS binding sites is similar to the conditions when binding was performed in the presence of additional $MgSO_4$. Due to the stable concentration of total binding sites, it may be considered unlikely that the addition of $MgSO_4$ would lead to a recruitment of new binding sites. An increase of the affinity of sites, already labeled by [^{3}H]TAPS, i.e. a change of a lower-affinity state of the same receptor towards a high-affinity conformation, may equally explain this effect. These data contrast results obtained by Standifer et al. (1993), showing that the Mg^{2+}-dependent increase of specific [^{3}H]DADLE binding at the putative MOP_1 binding site was due to an increase in both affinity and receptor density. When [^{3}H]TAPS is used as radioligand, the lower affinity in the absence of Mg^{2+} apparently is the result of an increase in the dissociation rate constants, while the association rate constants remain essentially stable under both conditions (Table 2).

The binding affinity of [^{3}H]TAPS was reduced in the presence of NaCl and Gpp(NH)p to an extent which was similar in the assays including K_2-EDTA or $MgSO_4$. Incubation with 100 mM NaCl alone led to a more pronounced reduction of the maximum receptor density in the presence of K_2-EDTA compared to the addition of 5 mM $MgSO_4$. Inclusion of Gpp(NH)p and NaCl resulted in an overall reduction of affinity. Moreover, exposure to NaCl appeared to reduce the maximum number of receptors, especially when other cations were removed by K_2-EDTA. The effect of Na^+ was reported earlier by others (Werling et al., 1986) to primarily affect the affinity of the MOP receptor and not the density of binding sites. This earlier finding stands in contrast to our results and may be due to the use of [^{3}H]DAMGO as radioligand and a different source of cerebral tissue (guinea pig). Interestingly, these authors reported at least two affinity states of the MOP receptor for DAMGO in NG 108-15

and 7315c cells (Werling et al., 1988) and guinea pig cortical membranes when Gpp(NH)p was added (Werling et al., 1984). Multiple receptor conformations resulting in different affinities of opioid agonists have also been identified for the putative MOP_1 receptor subtype (Standifer et al., 1993). In this latter study, [^{3}H]DADLE binding in the presence of blocking concentrations of DPDPE was augmented due to an increase of affinity and receptor number when 5 mM $MgSO_4$ was added. A third affinity state was proposed, which was induced by guanosine nucleotides and/or Na^+. The IC_{50} of Gpp(NH)p to inhibit [^{3}H]DADLE binding to MOP_1 receptors was about 30-fold higher in the absence than in the presence of $MgSO_4$, emphasizing the role of Mg^{2+} cations in the coupling of the G-protein–guanosine nucleotide complex to the receptor.

In competition experiments (Table 5), prototypical MOP-, DOP- and KOP agonists inhibited specific [^{3}H]TAPS binding, showing high affinities of MOP ligands, whereas DOP- and KOP-selective ligands were virtually inactive. When Mg^{2+} cations were removed by addition of K_2-EDTA, the affinities of MOP receptor-selective ligands were clearly reduced (6-fold). The change in affinity was most pronounced for DSTLE (16-fold). This finding is difficult to explain and may be due to some selectivity of DSTLE to the proposed MOP_1 receptor binding site, which appears more sensitive to Mg^{2+} than other classes of opioid receptors (Clark et al., 1988). On the other hand, the affinity shift of DADLE should then exhibit the same magnitude, which was not the case.

In conclusion, we characterized the receptor binding properties of TAPS, a dermorphin-derived tetrapeptide with differential pharmacological properties compared to morphine. A specific, MOP receptor-selective assay was developed using [^{3}H]TAPS as radioligand. TAPS bound to MOP receptors with differential affinities depending on the presence of Mg^{2+} cations. These observations may suggest different affinity states of the MOP receptor protein depending on ion concentrations. In the absence of cations, the affinity was reduced due to an increase of the dissociation rate, while the association rate was not significantly altered. The affinity was reduced when Na^+ and a stable GTP-analog, Gpp(NH)p, were added.

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