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Short communication

Binding of [^{3}H](+)-BW373U86 to δ-opioid receptors in rat brain membranes

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Abstract

A tritiated form of the non-peptidic δ-opioid receptor agonist (+)-BW373U86 ((+)-4-((α-*R*)-α-((2*S*,5*R*)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-hydroxybenzyl)-*N*,*N*-diethylbenzamide) was synthesized and its binding characteristics studied. [^{3}H](+)-BW373U86 bound with subnanomolar affinity to rat brain membranes and was displaced most effectively by ligands selective for δ-opioid receptors. Naltrindole, naltriben, and 7-benzylidenenaltrexone exhibited apparent inhibition constants of 0.06, 1.54, and 4.49 nM, respectively, while μ- or κ-selective ligands showed little affinity for this site. [^{3}H](+)-BW373U86 binding was sensitive to the presence of guanine nucleotides; GDP caused a 3-fold decrease and 5′-guanylyl-imidodiphosphate (Gpp[NH]p) caused a 25% increase in binding affinity.

Keywords: BW373U86; δ-Opioid receptor; G-protein; Radioligand binding

1. Introduction

Pharmacological studies carried out over the past two decades have implicated the existence of at least three subtypes of opioid receptor, termed μ, δ, and κ. The more recent cloning of receptors whose pharmacological profile matches those of the originally proposed opioid receptors supports this classification scheme (Evans et al., 1992; Chen et al., 1993; Meng et al., 1993). The study of individual subtypes of opioid receptors is made possible by ligands exhibiting selectivity for one subtype or another. In the case of the δ-opioid receptor, agonists such as [D-Pen^2,D-Pen^5]enkephalin (DPDPE), [D-Ala^2,D-Leu^5]enkephalin (DADLE), and the deltorphins, and antagonists such as naltrindole, naltriben, and 7-benzylidenenaltrexone have contributed greatly to the advances made in this field over the past few years (Mosberg et al., 1983; Sofuoglu et al., 1993). Until recently, the most highly selective δ-opioid receptor agonists were peptides. The first non-peptidic, δ-selective agonist to be introduced was (±)-4-((α-*R*)-α-((2*S*,5*R*)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-hydroxybenzyl)-*N*,*N*-diethylbenzamide (BW373U86) (Chang et al., 1993). In competition binding studies BW373U86 exhibited selectivity for δ-opioid receptors in purified brain membranes and in membranes isolated from the neuroblastoma/glioma cell line NG108-15 (Chang et al., 1993; Childers et al., 1993). Agonist activity of BW373U86 has been demonstrated by inhibition of adenylate cyclase in isolated membranes, by inhibition of electrically stimulated smooth muscle contraction in mouse vas deferens, and by several in vivo paradigms (Chang et al., 1993; Comer et al., 1993). To date, the pharmacological analysis of BW373U86 has been carried out using the racemic mixture of the compound. For the present study, we prepared tritiated BW373U86 and then purified the radiolabeled active (+)-enantiomer, [^{3}H](+)-BW373U86, to characterize directly the binding of this novel ligand in rat brain membranes. Our results fully support the δ-opioid receptor selectivity of this ligand. In addition, we demonstrated an increase in the binding affinity of the compound in the presence of Na^+ and 5′-guanylylimidodiphosphate (Gpp[NH]p). This atypical response is unique among δ-

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opioid receptor agonists and suggests a novel receptor/ligand interaction.

2. Materials and methods

Morphine, *trans*-(±)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)-cyclohexyl]-benzeneacetamide (U-50488), and naltriben were obtained from Research Biochemicals International (Natick, MA, USA). The peptide agonists DPDPE and deltorphin II were obtained from Peninsula Laboratories (Belmont, CA, USA). Naltrindole, 7-benzylidenenaltrexone, cyclic-D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2 (CTOP), (±)-4-((α-*R*)-α-((2*S*,5*R*)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl)-*N*,*N*-diethylbenzamide (BW932U90), and the (+)- and (−)-enantiomers of BW373U86 were synthesized at Burroughs Wellcome (Research Triangle Park, NC, USA). The synthesis of [^{3}H](+)-BW373U86 is described below. GDP and Gpp[NH]p were obtained from Sigma Chemical Company (St. Louis, MO, USA).

The preparation of [^{3}H](+)-BW373U86 was carried out as follows. BW373U86 (Bishop and McNutt, 1995) was de-allylated by heating at reflux for 3 days in 3:1 methanol/water containing 1% (v/v) trifluoroacetic acid and 5% palladium on carbon to give racemic (±)-4-((α*R**)-α-((2*S**,5*R**)-2,5-dimethyl-1-piperazinyl)-3-hydroxybenzyl)-*N*,*N*-diethylbenzamide. Prior to alkylating the piperazine nitrogen, the phenol was protected as the silyl ether with *tert*-butylchlorodimethylsilane and imidazole in *N*,*N*-dimethylformamide at room temperature. The resulting compound was purified by chromatography on silica gel (1–5% ethanol in dichloromethane) and alkylated with a slight excess of propargyl bromide and sodium carbonate in tetrahydrofuran at reflux overnight. The *t*-butyldimethyl-silyl protecting group was removed by treatment with tetraethylammonium fluoride in acetonitrile at room temperature (30 min) to give (±)-4-((α*R**)-α-((2*S**,5*R**)-2,5-dimethyl-4-(2-propynyl)-1-piperazinyl)-3-hydroxybenzyl)-*N*,*N*-diethylbenzamide. The propargyl analog of BW373U86 was dissolved in toluene with Lindlar's catalyst (5% palladium on calcium carbonate, poisoned with lead) and reduced under one atmosphere of tritium gas until the starting propargyl compound was > 50% consumed (high performance liquid chromatography (HPLC) analysis) [1]. The resulting mixture of starting material, desired (±)-[^{3}H]BW373U86, and over-reduced *N*-propyl compound was purified by preparative thin layer chromatography on silica gel (dichloromethane : ethanol : NH_4OH, 95 : 5 : 1). Radiochemical purity was 96.6% by HPLC radiodetection. The active (+)-enantiomer was purified by HPLC using a Cyclobond I column (Advanced Separation Technologies, Whippany, NJ, USA) and isocratic elution with 35% methanol : 65% 0.1 M ammonium acetate (v/v). Active fractions were identified by binding to rat brain membranes as described below.

Rat brains (minus cerebellum) were Dounce homogenized in ice-cold homogenization buffer consisting of 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 50 μg/ml soybean trypsin inhibitor, and 10 μg/ml each aprotinin and leupeptin. The particulate fraction was pelleted by centrifugation at 40 000 × *g* for 30 min at 4°C and resuspended in low-Tris homogenization buffer containing 5 mM Tris-HCl (pH 7.5), 1 mM EDTA, and protease inhibitors as above. Following a 30-min incubation on ice, the suspension was homogenized using an Ultra Turrax tissue disperser (IKA-Works, Cincinnati, OH, USA); unbroken cells and debris were removed by centrifugation at 500 × *g* for 10 min at 4°C. Membranes remaining in the supernatant fraction were recovered by centrifugation at 40 000 × *g* as before, resuspended in homogenization buffer, and stored at −70°C until use. Protein content was estimated by a dye-binding assay (BioRad, Richmond, CA, USA) using bovine serum albumin as standard.

Binding of [^{3}H](+)-BW373U86 in rat brain membranes was investigated by incubating membranes (100 μg protein/tube) with the radiolabeled ligand for 2 h at room temperature in a total volume of 1 ml. Incubations were carried out in 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 2 mg/ml bovine serum albumin, and protease inhibitors (binding buffer). The effect of Na^+ and GDP (100 mM and 100 μM, respectively) on binding was determined in binding buffer. The effect of Na^+ and Gpp[NH]p (100 mM and 50 μM, respectively) on binding was determined in 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 2 mg/ml bovine serum albumin, and protease inhibitors. Separation of bound from free ligand was accomplished by vacuum filtration through Whatman GF/C glass fiber filters (Whatman Paper, UK) using a Brandel model M-48 cell harvester (Brandel Research and Development Laboratories, Gaithersburg, MD, USA). The filters were then washed 3 times with 5 ml ice-cold 50 mM Tris-HCl (pH 7.5). Radioactivity associated with the membranes was quantitated by liquid scintillation spectrophotometry. For saturation binding experiments, binding at μ-opioid receptor sites was blocked by the inclusion of 2 μM CTOP in each tube. Specific binding was determined by subtracting the cpm bound in the presence of 1 μM naltriben from that bound in its absence.

3. Results

In previous studies, BW373U86 was shown to exhibit δ-opioid receptor-selective agonist activities in both in vitro and in vivo assays (Chang et al., 1993; Comer et al.,

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1993). In the present study, we performed [^{3}H](+)-BW373U86 saturation binding studies in rat brain membranes in the presence of 2 μM of the μ-opioid receptor antagonist CTOP. Under these conditions, [^{3}H](+)-BW373U86 bound to a single site with subnanomolar affinity (Table 1). We investigated the ligand selectivity of the [^{3}H](+)-BW373U86 binding site in rat brain membranes by means of competition binding assays. The displacement of [^{3}H](+)-BW373U86 binding by ligands selective for the various subclasses of opioid receptors is shown in Fig. 1. The most effective competitors for binding to this site were the δ-opioid receptor antagonists naltrindole, naltriben, and 7-benzylidenenaltrexone with apparent K_i values of 0.06 ± 0.003, 0.27 ± 0.2, and 1.54 ± 0.1 nM, respectively (Fig. 1A). The δ-opioid receptor-selective peptide agonists deltorphin II and DPDPE displaced [^{3}H](+)-BW373U86 binding with apparent K_i values of 4.49 ± 2.5 and 17.8 ± 3.4 nM, respectively, while the non-selective antagonist naloxone exhibited an apparent K_i of 22.6 ± 3.0 nM (data not shown). Morphine, on the other hand, was a much poorer competitor, with an apparent K_i of 219 ± 21 nM (Fig. 1A). CTOP and U-50488, ligands highly selective for μ- and κ-opioid receptors, respectively, showed little displacement even at 10 μM (Fig. 1A). The [^{3}H](+)-BW373U86 binding site showed strong stereoselectivity as non-radiolabeled (+)-BW373U86 displaced the binding of the tritiated compound with an apparent affinity of 0.23 ± 0.05 nM while that of the (−)-enantiomer was over two orders of magnitude higher at 29 ± 4.6 nM (Fig. 1B). BW932U90, a racemic mixture of a methylated form of BW373U86, exhibited a 10-fold lower affinity for the [^{3}H](+)-BW373U86 site than the parent compound with an apparent K_i of 2.64 ± 0.08 nM.

Given that opioid receptors belong to the family of G-protein coupled receptors, we investigated the effect of Na^+ and guanine nucleotides on [^{3}H](+)-BW373U86 binding. The inclusion of Na^+ and GDP in the binding reaction, conditions predicted to shift the receptor to a low affinity state, resulted in a 3-fold decrease in binding affinity. Conversely, the addition of Na^+ and Gpp[NH]p caused a 25% increase in [^{3}H](+)-BW373U86 binding affinity when measured in the presence of 5 mM Mg^{2+} (Table 1).

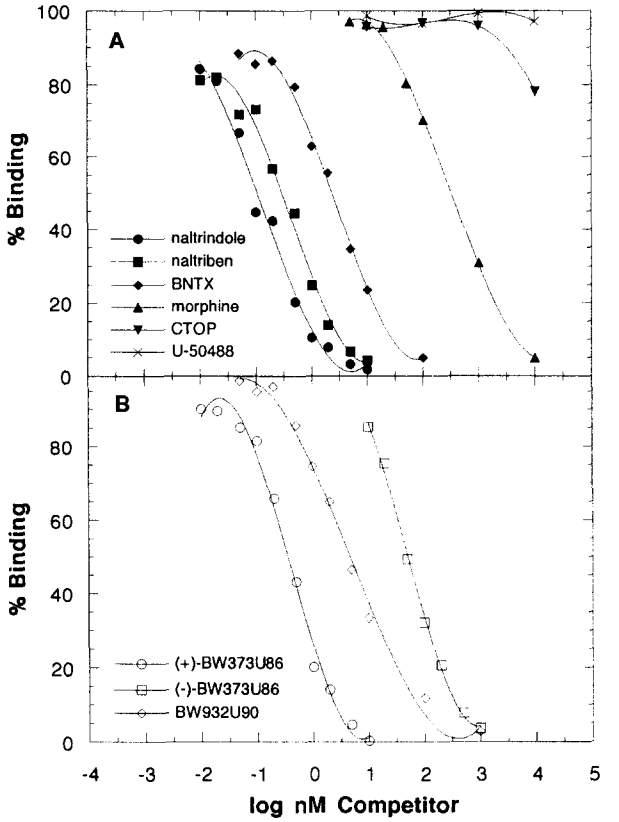

Fig. 1. Competition binding studies of [^{3}H](+)-BW373U86 in rat brain membranes. Binding of [^{3}H](+)-BW373U86 in rat brain membranes was carried out as described in Materials and methods. Incubations were carried out in the presence or absence of the indicated concentrations of non-radiolabeled competitor. Plot A depicts the selectivity of the [^{3}H](+)-BW373U86 binding site for δ- (naltrindole, naltriben, 7-benzylidenenaltrexone (BNTX)), μ- (morphine, CTOP), or κ- (U-50488) opioid receptor ligands. Plot B depicts the selectivity of the site for non-radiolabeled (+)-BW373U86, the less active (−)-enantiomer (−)-BW373U86, or an *O*-methylated derivative BW932U90. The apparent inhibition constants (K_i) reported in the text are the mean ± S.D. of 3 experiments and were calculated using the method of Cheng and Prusoff (1973).

Table 1
Binding of [^{3}H](+)-BW373U86 in rat brain membranes

	K_D (nM)	B_{max} (fmol/mg protein)
Control	0.13 ± 0.007	200 ± 2.1
Na^+/GDP	0.35 ± 0.014 [a]	165 ± 2.2 [b]
$MgCl_2$	0.44 ± 0.028 [b]	217 ± 4.7
$MgCl_2$/Na^+/Gpp[NH]p	0.33 ± 0.021 [c]	162 ± 18 [c]

Binding was carried out as described in Materials and methods. Dissociation constants (K_D) and B_{max} values were calculated directly from saturation binding data using the computer program PRISM (GraphPad Software, San Diego, CA, USA). Data are expressed as the mean ± S.E. of thee experiments. [a] $P < 0.01$ cf. control; [b] $P < 0.001$ cf. control; [c] $P < 0.01$ cf. $MgCl_2$ (Student's *t*-test).

4. Discussion

The results reported here, obtained using the tritiated active (+)-enantiomer, confirm the δ-opioid receptor selectivity of BW373U86. [^{3}H](+)-BW373U86 bound to a single site in rat brain membranes and was most effectively displaced by ligands of known δ-opioid receptor selectivity. Furthermore, the affinity of [^{3}H](+)-BW373U86 for its binding site in rat brain membranes is indistinguishable from that measured in the mouse vas deferens isolated tissue assay (Chang et al., 1993), a system recognized to represent δ-opioid receptor activity (Wild et al., 1993b).

Work over the past several years has provided evidence for the existence of at least two subtypes of δ-opioid receptor, termed δ_1 and δ_2 (Sofuoglu et al., 1993). Additional studies have shown that rat brain and mouse vas deferens δ-opioid receptors are different; the data favor the existence of the δ_2 subtype in vas deferens (Vaughn et al., 1990; Wild et al., 1993a). Our results in rat brain membranes show that the δ_2-opioid receptor-selective antagonist naltriben has a higher affinity for the [^{3}H](+)-BW373U86 binding site than does the δ_1-opioid receptor-selective antagonist 7-benzylidenenaltrexone (Fig. 1). Likewise, the δ_2-opioid receptor-selective peptide agonist deltorphin II has a higher affinity than does the δ_1-opioid receptor-selective agonist DPDPE. These results, in combination with the extremely high potency of BW373U86 in the mouse vas deferens assay, are consistent with the binding site being the δ_2-opioid receptor.

Agonist binding to G-protein-coupled receptors is sensitive to the presence of Na^+ and GTP or Gpp[NH]p (Wild et al., 1993b; Wong et al., 1994). This so-called 'G-shift' is believed to be the result of the dissociation of the receptor from the GTP-bound G-protein. A detailed study of this phenomenon provides evidence for a complex interaction between G-protein and receptor whereby Na^+, Mg^{2+}, GTP, GDP, and the agonist interact in various combinations to regulate the affinity state of the receptor (Wong et al., 1994). In their model, Na^+ plus GDP, or Na^+ plus GTP in the presence of Mg^{2+}, results in a low affinity form of the receptor. Our data, showing a nearly 3-fold decrease in binding affinity in the presence of Na^+ and GDP, support this model. However, when measured in the presence of Mg^{2+}, we find that Na^+ and Gpp[NH]p actually increase the affinity of [^{3}H](+)-BW373U86 for its receptor in rat brain membranes by approximately 25% (Table 1). A lack of sensitivity of BW373U86 to Na^+ and Gpp[NH]p has been reported (Childers et al., 1993). We feel that these data are not necessarily at variance with ours as the small increase in binding affinity we observed may only be detectable by directly measuring the binding of the radiolabeled active (+)-enantiomer. Although our results support a functional coupling between the [^{3}H](+)-BW373U86 receptor and G-proteins, the coupling appears to be different from that observed for other δ-opioid receptor agonists. A recent report by Li et al. (1995) shows that SNC121, a methylated derivative of [^{3}H](+)-BW373U86, requires the Trp^{284} amino acid residue of the human δ-opioid receptor for binding whereas the highly δ-opioid receptor-selective agonists deltorphin II and [4′-*p*-Cl-Phe^4]DPDPE do not. It is possible that this difference between the ligand binding pockets of SNC121 or [^{3}H](+)-BW373U86 and the peptide agonists is the basis for the variance in the receptor/G-protein coupling observed with these two groups of compounds.

In summary, the data presented above demonstrate that [^{3}H](+)-BW373U86 binds with subnanomolar affinity to a single site in rat brain membranes. Competition binding assays strongly suggest that this site is the δ-opioid receptor, possibly the δ_2 subtype. The binding of [^{3}H](+)-BW373U86 to this site in rat brain membranes is affected by the presence of Na^+ and guanine nucleotides, implying an interaction with G-proteins. The affinity shifts observed, however, are uncharacteristic of classic δ-opioid receptor agonists, possibly indicating a novel binding of [^{3}H](+)-BW373U86 to this receptor.

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