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Several δ-opioid receptor ligands display no subtype selectivity to the human δ-opioid receptor

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Abstract

Pharmacological studies performed in vivo suggested that the δ-opioid receptor could exist as two distinct subtypes, δ_1 and δ_2, while in vitro studies are inconclusive. Therefore, we measured the binding and functional selectivity of several putative δ_1- and δ_2-opioid receptor-selective compounds in membranes from Chinese hamster ovary cells stably expressing the human δ-opioid receptor. The compounds characterized were the agonists [D-Pen^2,D-Pen^5]enkephalin (DPDPE, δ_1) and deltorphin II (δ_2), and the antagonists 7-benzylidenenaltrexone (BNTX, δ_1), naltriben (δ_2), naltrindole 5′-isothiocyanate (δ_2), and naltrindole (δ_1 and δ_2). In competition binding assays, all compounds tested showed no preference for the [^{3}H]DPDPE, [^{3}H]deltorphin II, or [^{3}H]naltrindole binding sites. BNTX also showed no selectivity for the δ-opioid receptor over the μ-opioid receptor. In functional assays, the stimulation of [^{35}S]GTPγS binding induced by either DPDPE or deltorphin II was potently inhibited by both δ_1- and δ_2-opioid receptor-selective antagonists. Together, these results indicate that these compounds are not selective for either the δ_1- or δ_2-opioid receptor binding sites in binding or functional assays.

Keywords: δ-Opioid receptor, subtype; Chinese hamster ovary (CHO) cell; Binding; [^{35}S]GTPγS; Naltrindole derivative

1. Introduction

Pharmacological and molecular cloning studies have identified three major opioid receptor types, namely μ, δ, and κ. Although only these three receptors have been cloned, there is evidence that each receptor does not exist as a homogenous population. Approximately 10 years ago, the concept of multiple δ-opioid receptor subtypes arose (for a thorough review, see Traynor and Elliot, 1993; Zaki et al., 1996). The first indication for δ-opioid receptor subtypes came from mice antinociception studies. Researchers found that pretreatment of mice with the noncompetitive δ-opioid receptor antagonist, [D-Ala^2, Leu^5, Cys^6]enkephalin (DALCE), could antagonize the antinociceptive effects of [D-Pen^2, D-Pen^5]enkephalin (DPDPE), but not deltorphin II (Jiang et al., 1991). Conversely, another noncompetitive δ-opioid receptor antagonist, naltrindole 5′-isothiocyanate (NTII), blocked the antinociceptive effects of deltorphin II but not DPDPE (Jiang et al., 1991).

The development of competitive, alkaloid δ-opioid receptor-selective compounds by Portoghese et al. (1988a, 1991, 1992) further strengthened the hypothesis of δ-opioid receptor subtypes. Naltriben, the benzofuran derivative of the potent δ-opioid receptor antagonist naltrindole (Portoghese et al., 1988b), had subnanomolar affinity for the δ-opioid receptor. Naltriben antagonized the antinociceptive effects of [D-Ser^2, Leu^5, Thr^6]enkephalin (DSLET), but not the effects of DPDPE (Sofuoglu et al., 1991). Conversely, 7-benzylidenenaltrexone (BNTX), an analog of the nonselective-opioid receptor antagonist naltrexone, antagonized the antinociceptive effects of DPDPE but not the effects of DSLET (Portoghese et al., 1992; Sofuoglu et al., 1993). In addition, pretreatment with DPDPE did not produce tolerance to the antinociceptive effect of deltorphin II, indicating that these agonists may elicit their effects through discrete opioid receptor subtypes (Mattia et al., 1991). The above observations, along with other evidence, led to the classification of the δ_1- and δ_2-opioid receptor subtypes (Zaki et al., 1996).

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The δ_1-opioid receptor subtype is classified as the subtype that preferentially binds DPDPE, DALCE, and BNTX and the δ_2-opioid receptor subtype is classified as the subtype that preferentially binds to deltorphin II, DSLET, NTII, and naltriben.

Although the in vivo selectivity of these compounds is convincing, in vitro studies are not as persuasive. Radioligand binding studies performed with rat brain homogenates (Negri et al., 1991) and membranes isolated from the human neuroblastoma cell line SK-N-BE (Allouche et al., 2000) indicated that two distinct δ-opioid binding sites exist. However, studies with membranes from another human neuroblastoma cell line, SH-SY5Y, only identified a single δ-opioid receptor binding site (Toll et al., 1997). Furthermore, the δ_2-opioid receptor-selective antagonist, naltriben, displayed equal affinity in displacing [^{3}H]DPDPE, [^{3}H][pCl-Phe^4]DPDPE, [^{3}H]DSLET, [^{3}H]deltorphin II, and [^{3}H][Ile^{5,6}]deltorphin II in mouse (Sofuoglu et al., 1992), rat (Buzas et al., 1994), and human (Kim et al., 2001) brain membranes. Yet, BNTX, the δ_1-opioid receptor-selective antagonist, had higher affinity competing with [^{3}H]DPDPE compared to tritiated-δ_2-opioid receptor ligands in rat (Buzas et al., 1994), guinea pig (Portoghese et al., 1992), and human (Kim et al., 2001) brain membranes.

In studies with SH-SY5Y neuroblastoma cells (Toll et al., 1997) and Chinese hamster ovary (CHO) cells stably expressing the cloned mouse δ-opioid receptor (Raynor et al., 1994), researchers observed that both naltriben and BNTX had high affinities for the binding site of the non-selective δ-opioid receptor antagonist, [^{3}H]naltrindole, and the binding site of the δ_1-opioid receptor-selective agonist, [^{3}H][pCl-Phe^4]DPDPE. However, the affinity of naltriben is considerably higher than that of BNTX, and this led to the conclusion that the δ-opioid receptor expressed on SH-SY5Y cells and the cloned mouse receptor could classify as being the δ_2-opioid receptor subtype.

Characterizing the functional selectivity of these ligands in vitro has been challenging. Researchers observed that in rat brain homogenates, naltriben preferentially reduced deltorphin II and DSLET-induced inhibition of adenylyl cyclase activity and that BNTX preferentially reduced the inhibition produced by DPDPE (Buzas et al., 1994). However, in SH-SY5Y cells, naltriben inhibited DPDPE- and deltorphin II-induced inhibition of adenylyl cyclase activity with the same potency (Toll et al., 1997). Yet, BNTX, which is considerably less potent than naltriben, preferentially inhibited DPDPE-induced inhibition of adenylyl cyclase in these cells (Toll et al., 1997). In another assay, both naltriben and BNTX potently inhibited DPDPE-induced stimulation of [^{35}S]GTPγS binding in NG108-15 cells (Szekeres and Traynor, 1997), which endogenously express the δ-opioid receptor, and in C_6 glioma cells stably transfected with the cloned rat δ-opioid receptor (Clark et al., 1997). In the C_6 glioma cell model, BNTX, naltriben, and naltrindole were equipotent at inhibiting this DPDPE-induced stimulation. Finally, in SH-SY5Y cells, researchers found no evidence of subtype selectivity in the opioid-induced increase in intracellular calcium after carbachol stimulation (Connor et al., 1997).

The aim of this study was to further characterize the selectivity of the δ_1- and δ_2-opioid receptor agonists and antagonists using membranes from CHO cells that stably expressed the human δ-opioid receptor and to directly compare these compounds with the prototypical δ-opioid receptor antagonist, naltrindole. Therefore, we studied the binding of the putative δ_1-opioid receptor-selective agonist [^{3}H]DPDPE, the putative δ_2-opioid receptor-selective agonist [^{3}H]deltorphin II, and the nonselective δ-opioid receptor antagonist [^{3}H]naltrindole. Due to the colocalization of μ and δ receptors in many regions of the brain (Simon and Hiller, 1994), it was also important to test if these δ-opioid receptor-subtype-selective ligands had any affinity for the μ-opioid receptor. For that reason, the μ-opioid receptor-selective agonist [^{3}H][D-Ala^2, N-Me, Phe^4, glycinol^5]enkephalin (DAMGO) was also studied in binding assays. In functional assays, δ_1-(BNTX) and δ_2-(naltriben and NTII) opioid receptor-selective antagonists and a nonsubtype-selective δ-opioid receptor antagonist (naltrindole) were titrated in order to study their effectiveness at inhibiting both DPDPE- and deltorphin II-induced stimulation of [^{35}S]GTPγS binding.

2. Materials and methods

2.1. Cell culture

CHO cells, stably transfected with the human δ-opioid receptor (hDOR-CHO) and the μ-opioid receptor (hMOR-CHO), were obtained from Drs. Larry Toll (SRI International, Palo Alto, CA, USA) and George Uhl (NIDA Intramural Program, Baltimore, MD, USA), respectively. The cells were grown in 100-mm dishes in Dulbecco's modified Eagle's media (DMEM) supplemented with 10% fetal bovine serum and penicillin–streptomycin (10,000 units/ml) at 37 °C in a 5% CO_2 atmosphere.

2.2. Membrane preparation

Upon reaching 90–95% confluence, cells were harvested from dishes by scraping in DMEM followed by centrifugation at 200 × *g* for 10 min. The cells were resuspended in phosphate-buffered saline (PBS), pH 7.4, containing 0.04% EDTA and centrifuged again at 200 × *g* for 10 min. Following this centrifugation, the pellet was resuspended in 50 mM Tris–HCl, pH 7.5 for receptor binding assays or in a buffer of 50 mM Tris–HCl, 3 mM $MgCl_2$, 1 mM EGTA, pH 7.4 (membrane buffer) for [^{35}S]GTPγS binding assays. After homogenization, the resulting mixture was spun at 18,800 × *g* for 30 min. The supernatant was discarded and the pellet was resuspended in 50 mM Tris–HCl, pH 7.5 or membrane buffer. This suspension was centrifuged at

18,800 × *g* for 30 min. The final pellet was resuspended in 50 mM Tris–HCl, pH 7.5 for binding assays or in a buffer containing 50 mM Tris–HCl, 3 mM $MgCl_2$, 100 mM NaCl, 0.2 mM EGTA, pH 7.4 (assay buffer) for [^{35}S]GTPγS binding assays. Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as the standard. Membranes were aliquoted into cryovials and stored at −80 °C.

2.3. Receptor binding assays

The radioligands used were [^{3}H]deltorphin II, [^{3}H]-DPDPE, [^{3}H]naltrindole, and [^{3}H]DAMGO. Binding was performed in 50 mM Tris–HCl, pH 7.5, at 25 °C for 60 min for [^{3}H]deltorphin II and [^{3}H]DAMGO, 2 h for [^{3}H]DPDPE, and 3 h for [^{3}H]naltrindole. Membrane protein (20 μg hDOR-CHO and 50 μg hMOR-CHO) was incubated with radiolabeled ligand in the presence of varying concentrations of δ-opioid receptor ligands. Nonspecific binding was determined in the presence of 10 μM naloxone. Incubations were performed in triplicate at a final volume of 1 ml. The binding reaction was terminated via rapid filtration though Schleicher and Schuell No. 32 glass fiber filters using a 48-well Brandel cell harvester. Filters were rinsed three times with 3 ml of ice-cold 50 mM Tris–HCl, pH 7.5 and subsequently counted in 2 ml of EcoScint A scintillation fluid for 2 min.

2.4. [^{35}S]GTPγS binding assay

Prepared hDOR-CHO membrane protein (15 μg) was incubated in assay buffer containing 0.08 nM [^{35}S]GTPγS, 3 μM GDP, and varying concentrations of δ-opioid receptor ligands. Nonspecific binding was defined using 10 μM unlabeled GTPγS. The mixture was incubated at 30 °C for 60 min at a final volume of 0.5 ml. The reaction was terminated via rapid filtration through a glass fiber filter as described above. Filters were also rinsed and counted as indicated above.

2.5. Chemicals and drugs

[^{3}H]Deltorphin II (41.0 Ci/mmol), [^{3}H]naltrindole (35.0 Ci/mmol), [^{3}H]DPDPE (35 Ci/mmol), and [^{35}S]GTPγS (1250 Ci/mmol) were purchased from Perkin-Elmer Life Sciences (Boston, MA, USA), and [^{3}H]DAMGO (64.0 Ci/mmol) was obtained from Amersham Biosciences (Piscataway, NJ, USA). Deltorphin II and DPDPE were from Bachem (Torrance, CA, USA). NTII and naltrindole were purchased from Sigma-Aldrich (St. Louis, MO, USA). BNTX and naltriben were purchased from Tocris-Cookson (Ballwin, MO, USA). DMEM, fetal bovine serum and penicillin–streptomycin were obtained from Life Technologies (Grand Island, NY, USA). EcoScint A was purchased from National Diagnostic (Atlanta, GA, USA). All other chemicals were purchased from Sigma-Aldrich.

2.6. Statistics

The K_i values of unlabeled compounds were calculated from the equation $K_i = IC_{50}/[1+(\text{concentration of radioligand}/K_d \text{ of radioligand})]$ (Cheng and Prusoff, 1973). The K_d values for the binding of [^{3}H]deltorphin II, [^{3}H]naltrindole, [^{3}H]DPDPE, and [^{3}H]DAMGO were 4.49, 0.096, 1.30, and 0.531 nM, respectively. The EC_{50} values for stimulation of [^{35}S]GTPγS binding were determined by nonlinear curve fitting of dose response curves using Sigma Plot (Jandel Scientific, San Rafael, CA). Apparent antagonist affinity constants (K_e) were calculated as previously described (Szekeres and Traynor, 1997). Briefly, the values were calculated from [^{35}S]GTPγS binding curves in the presence or absence of single concentration of antagonist. The equation used was, K_e=[antagonist]/[(EC_{50} in the presence of antagonist/EC_{50} in the absence of antagonist) − 1]. Students *t*-tests were used for statistical comparisons between groups of data where $P < 0.05$ was considered significant.

3. Results

3.1. Binding profile of putative δ_1- and δ_2-opioid receptor ligands to the human δ- and μ-opioid receptors

K_i values for several δ-opioid receptor-selective compounds were determined by the inhibition of the binding of [^{3}H]deltorphin II, [^{3}H]naltrindole, and [^{3}H]DPDPE to hDOR-CHO membranes and by the inhibition of the binding of [^{3}H]DAMGO to hMOR-CHO membranes (Table 1). As expected, the putative δ_2-opioid receptor-selective agonist, deltorphin II, displayed high affinity in inhibiting [^{3}H]deltorphin II binding and the binding of the nonselective δ-opioid receptor antagonist, [^{3}H]naltrindole. However, it also had very high affinity in competing with the δ_1-opioid receptor-selective ligand, [^{3}H]DPDPE. It had very low affinity in inhibiting the μ-opioid receptor-selective ligand [^{3}H]DAMGO in hMOR-CHO membranes. DPDPE, the putative δ_1-opioid receptor-selective agonist, also showed very low affinity for the μ-opioid receptor. However, it displayed near equal affinity in inhibiting the binding of the δ_2-opioid receptor-selective agonist, [^{3}H]deltorphin II, as compared to the δ_1-opioid receptor selective agonist, [^{3}H]DPDPE, and slightly lower affinity in competing with the nonselective δ-opioid receptor antagonist, [^{3}H]naltrindole.

BNTX, a δ_1-opioid receptor antagonist, inhibited [^{3}H]-DPDPE, [^{3}H]deltorphin II, and [^{3}H]naltrindole binding with high affinity. It had slightly higher affinity at the [^{3}H]DPDPE and [^{3}H]deltorphin II binding sites compared to the [^{3}H]naltrindole binding site. Surprisingly, it displayed an equal affinity for the μ-opioid receptor (Table 1). Naltriben had very high affinity in competing with [^{3}H]deltorphin II, [^{3}H]DPDPE, and [^{3}H]naltrindole, and these

Table 1
K_i values for the inhibition of [^{3}H]DPDPE, [^{3}H]deltorphin II, [^{3}H]naltrindole, and [^{3}H]DAMGO binding to the human δ-opioid receptor and μ-opioid receptor by various δ ligands

Compound	K_i (nM) ± S.E.M.			
	[^{3}H]DPDPE	[^{3}H]Deltorphin II	[^{3}H]Naltrindole	[^{3}H]DAMGO
DPDPE	1.14 ± 0.205	2.01 ± 0.518	4.58 ± 0.566	269 ± 39.4
BNTX	1.08 ± 0.140	1.79 ± 0.121	2.67 ± 0.205	2.47 ± 0.090
Deltorphin II	1.74 ± 0.141	4.09 ± 0.227	6.67 ± 0.461	667 ± 191
Naltriben	0.155 ± 0.030	0.438 ± 0.055	0.382 ± 0.004	21.5 ± 1.12
NTII[a]	2.72 ± 0.282	5.36 ± 0.178	5.20 ± 0.402	16.0 ± 1.47
Naltrindole	0.232 ± 0.010	0.394 ± 0.072	0.389 ± 0.026	5.17 ± 0.884

Affinities were determined by inhibition of specific tritiated ligand binding to membranes of hDOR-CHO ([^{3}H]DPDPE, [^{3}H]deltorphin II, and [^{3}H]naltrindole) and hMOR-CHO ([^{3}H]DAMGO) cells in Tris–HCl buffer as described in Materials and methods. Data are expressed as mean K_i values ± S.E.M. from at least three experiments performed in triplicate.

[a] K_i values reported are apparent due to the covalent binding properties of NTII.

affinities were nearly equal to those of its parent compound, naltrindole. However, naltriben was more selective than naltrindole for the δ-opioid receptor. Naltriben was 50 times more selective for the δ-opioid receptor than the μ-opioid receptor and naltrindole was only 12 times more selective for the δ-opioid receptor than the μ-opioid receptor. Another δ_2-opioid receptor antagonist, NTII, showed equal apparent affinity in inhibiting [^{3}H]deltorphin II and [^{3}H]naltrindole binding and slightly better affinity at blocking [^{3}H]DPDPE binding. Yet, it was only three times more selective for the δ-opioid receptor than the μ-opioid receptor.

3.2. Inhibition of δ-opioid receptor agonist-stimulated [^{35}S]GTPγS binding by δ-opioid receptor antagonists

Receptor binding experiments showed that both δ_1- and δ_2-opioid receptor-selective compounds bound with high affinity to the [^{3}H]deltorphin II and [^{3}H]DPDPE binding sites. In order to test if this lack of subtype binding selectivity correlated with a lack of functional selectivity, we assayed the inhibition of DPDPE- and deltorphin II-stimulation of [^{35}S]GTPγS binding by BNTX, naltriben, NTII, and naltrindole.

DPDPE and deltorphin II were equally efficacious and similarly potent at stimulating [^{35}S]GTPγS binding in hDOR-CHO membranes. EC_{50} values were 14.7 ± 4.03 nM for DPDPE and 6.92 ± 0.80 nM for deltorphin II. Both compounds reached maximal stimulation at 1 μM and the E_{max} values for DPDPE and deltorphin II were 154 ± 16.2% and 134 ± 15.9%, respectively. At the δ-opioid receptor, 200 nM of either agonist stimulated [^{35}S]GTPγS binding to 70–80% of its E_{max} value. This concentration of agonist was used to characterize the potency of various compounds at inhibiting δ_1- and δ_2-opioid receptor agonist-stimulated [^{35}S]GTPγS binding.

Stimulation by a concentration of 200 nM of either DPDPE or deltorphin II was inhibited by δ_1-opioid receptor-selective, δ_2-opioid receptor-selective, and nonselective δ-opioid receptor compounds (Fig. 1 and Table 2). The IC_{50} values were in the nanomolar range as expected from the binding affinities. As shown in Table 2, NTII, naltriben, and BNTX were twice as effective in inhibiting DPDPE-stimulated [^{35}S]GTPγS binding than deltorphin II-stimu-

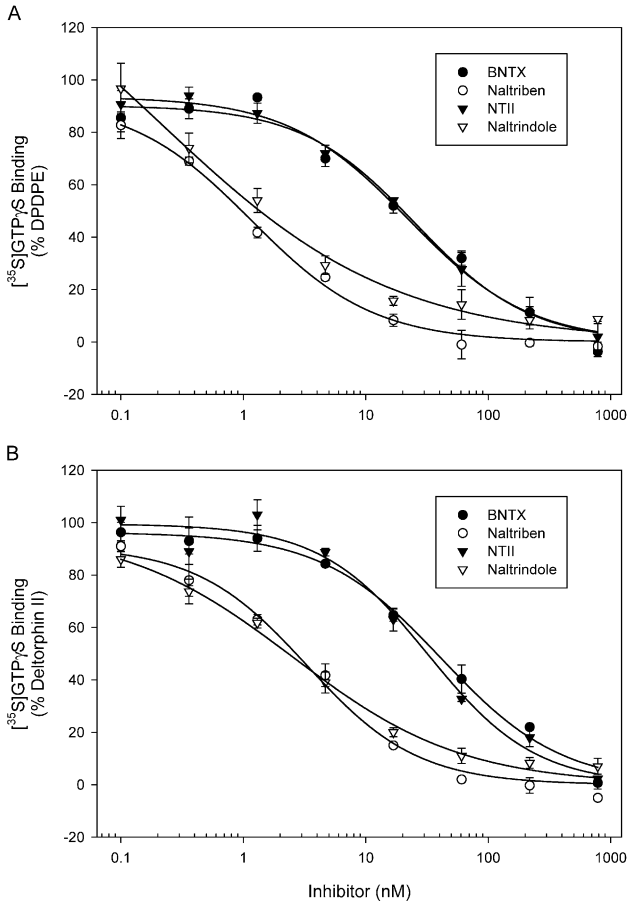

Fig. 1. Inhibition of DPDPE (A) and Deltorphin II (B)-stimulated [^{35}S]GTPγS binding to hDOR-CHO membranes by BNTX, naltriben, NTII, and naltrindole. Data are shown as percent stimulation produced by 200 nM of the agonist. DPDPE and deltorphin II alone stimulated [^{35}S]GTPγS binding by 106 ± 2.60% and 107 ± 4.40%, respectively, in hDOR-CHO membranes. Values represent the mean ± S.E.M. from three separate experiments, performed in triplicate.

Table 2
Inhibition of δ-opioid receptor agonist-stimulated [^{35}S]GTPγS binding by δ-opioid receptor antagonists

Agonist	IC_{50} (nM) ± S.E.M.			
	BNTX	NTII	Naltriben	Naltrindole
DPDPE	19.0 ± 2.32	18.1 ± 2.12	0.963 ± 0.138	1.57 ± 0.439
Deltorphin II	38.2 ± 4.24	35.4 ± 1.84	2.24 ± 0.403	2.08 ± 0.314

IC_{50} values are defined as the concentration of antagonist that inhibited 50% of 0.08 nM [^{35}S]GTPγS binding in the presence of 200 nM DPDPE or deltorphin II. This concentration of DPDPE and deltorphin II stimulated binding by 106 ± 2.60% and 107 ± 4.40%, respectively, over basal levels. Each antagonist was assayed at eight different concentrations (0.1–1000 nM). Data are listed as the mean IC_{50} values ± S.E.M. from three or more experiments, performed in triplicate.

Table 3
Shift in DPDPE and deltorphin II EC_{50} values in stimulating [^{35}S]GTPγS binding by BNTX and naltriben

Agonist		EC_{50} (nM) ± S.E.M.		
		Control	+10 nM BNTX	+1 nM naltriben
DPDPE		14.7 ± 4.03	86.7 ± 13.0[a]	156 ± 31.8[a]
	K_e	–	2.10 ± 0.530	0.113 ± 0.041
Deltorphin II		6.90 ± 0.80	17.9 ± 0.07[a]	54.7 ± 9.45[a]
	K_e	–	6.45 ± 1.17	0.168 ± 0.055

Agonist was titrated in the presence or absence of a set concentration of antagonist. Data are expressed as the mean value ± S.E.M. from at least three experiments performed in triplicate. K_e values were determined as described in Materials and methods.
[a] $P < 0.05$ compared to agonist alone.

lated binding. Naltriben and naltrindole were approximately 20-fold more potent than BNTX and NTII at inhibiting both DPDPE- and deltorphin II-induced stimulation of [^{35}S]GTPγS binding. Similar to what was seen in receptor binding assays, the IC_{50} values for naltriben were very close to that of its parent compound, naltrindole. Additionally, and most striking, naltriben inhibited DPDPE-induced stimulation of [^{35}S]GTPγS binding as well as deltorphin II-induced stimulation. These results indicate that, in hDOR-CHO membranes, there is no functional selectivity of naltriben in inhibiting δ_2-opioid receptor ligand-induced responses over δ_1-opioid receptor ligand-induced responses.

3.3. Shift in EC_{50} values caused by coadministration of δ_1- and δ_2-opioid receptor antagonists

As shown in Fig. 1 and Table 2, BNTX and naltriben antagonized the stimulatory effect of both DPDPE and deltorphin II. In order to calculate apparent K_e values for these two compounds, we titrated either DPDPE or deltorphin II in the presence or absence of a single concentration of antagonist (10 nM BNTX and 1 nM naltriben).

Naltriben shifted the dose–effect curve of DPDPE approximately 12-fold to the right giving an apparent K_e value of 0.113 ± 0.041 nM. It was slightly less potent at antagonizing the stimulatory effect of deltorphin II, producing a 9-fold shift in the concentration-effect curve and a K_e value of 0.168 ± 0.055 nM. As indicated in Table 2, BNTX was much less potent than naltriben at inhibiting the stimulatory effect of both DPDPE and deltorphin II. Yet, it showed marked selectivity for inhibiting DPDPE-induced stimulation of [^{35}S]GTPγS binding over deltorphin II-induced stimulation. BNTX produced approximately a 7-fold shift in the dose–effect curve for DPDPE, producing a K_e value of 2.10 ± 0.530 nM, and a 3-fold shift in the deltorphin II concentration-effect curve with a K_e value of 6.45 ± 1.17 nM (Fig. 2 and Table 3).

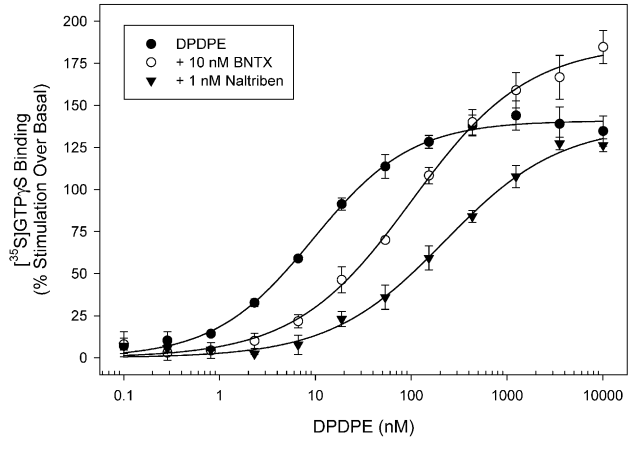

Fig. 2. Stimulation of [^{35}S]GTPγS binding to hDOR-CHO membranes by DPDPE in the presence or absence of BNTX (10 nM) and naltriben (1 nM). Values represent the mean ± S.E.M. from three separate experiments performed in triplicate.

4. Discussion

The evidence for δ-opioid receptor heterogeneity in vivo is convincing, yet the cellular and biochemical causes of this heterogeneity have not been established. In this current study, the receptor binding data clearly indicated that none of the unlabeled compounds tested were significantly selective for the δ_1- or δ_2-opioid receptor binding sites (Table 1). The lack of selectivity was two-sided as the putative δ_1-opioid receptor-selective compounds (DPDPE and BNTX) had high affinity for the δ_2-opioid receptor binding site, and the putative δ_2-opioid receptor-selective compounds (deltorphin II, naltriben, and NTII) had high affinity for the δ_1-opioid receptor binding site. The slight increase in affinity of BNTX and DPDPE for the [^{3}H]DPDPE binding site compared to the [^{3}H]deltorphin II binding site likely does not indicate an increase in selectivity for the δ_1-opioid receptor binding site because putative δ_2-opioid receptor-selective compounds also had higher affinity at this binding site compared to the [^{3}H]deltorphin II binding site. The rank orders of affinity of the ligands in displacing [^{3}H]DPDPE and [^{3}H]deltorphin II binding are similar with those

obtained from SH-SY5Y cells (Toll et al., 1997). Corresponding with our data, researchers found that δ_2-opioid receptor-selective compounds were equally potent at competing with [^{3}H][pCl-Phe^4]DPDPE as δ_1-opioid receptor-selective compounds (Toll et al., 1997). Studies with human brain membranes showed that deltorphin II and naltriben were not selective for the [^{3}H][Ile^{5,6}]deltorphin II binding site over the [^{3}H]DPDPE binding site (Kim et al., 2001). Yet, in contrast to our results, they showed a small selectivity of DPDPE and BNTX for the [^{3}H]DPDPE binding site. This discrepancy is likely because they used a different δ_2-opioid receptor radioligand and human brain membranes instead of cell membranes from a stably transfected cell line.

One of the most surprising findings was the lack of δ-μ-opioid receptor selectivity of several compounds. Most striking was BNTX, which showed nearly equal affinity for the [^{3}H]DPDPE, [^{3}H]deltorphin II, [^{3}H]naltrindole, and [^{3}H]DAMGO binding sites (Table 1). Yet, early studies showed that BNTX displayed selectivity for the δ-opioid receptor over the μ-opioid receptor in smooth muscle preparations (Portoghese et al., 1992). In vivo, BNTX had no effect on DAMGO- or morphine-induced antinociception in mice when administered intrathecally or subcutaneously (Sofuoglu et al., 1993). However, more recent work in vivo correlates with our binding results in that BNTX affects μ-mediated antinociception in mice by shifting the dose–response curve of DAMGO 4-fold to the right (Hammond et al., 1995).

In functional assays, δ_1-opioid receptor-selective DPDPE and δ_2-opioid receptor-selective deltorphin II were equally efficacious at stimulating [^{35}S]GTPγS binding, which corresponds with previous studies (Clark et al., 1997). Also, we demonstrated that DPDPE and deltorphin II have nearly equal potency in stimulating [^{35}S]GTPγS binding. Corresponding with our receptor binding data, δ_1- and δ_2-opioid receptor antagonists inhibited responses induced by both DPDPE and deltorphin II. Naltriben was more potent than BNTX at inhibiting the activation of the δ-opioid receptor induced by both DPDPE and deltorphin II (Table 2, Fig. 1), which is comparable to previous studies (Toll et al., 1997). Naltriben had an IC_{50} value that was approximately equal to its parent compound, naltrindole. BNTX and NTII were substantially less effective at inhibiting DPDPE- and deltorphin II-induced stimulation of [^{35}S]GTPγS binding as they had IC_{50} values that were approximately 20-fold higher than naltrindole and naltriben. In experiments with a set concentration of antagonist and several concentrations of agonist (Table 3, Fig. 2), BNTX showed selectivity for inhibiting DPDPE responses over deltorphin II responses. Naltriben was also more potent at inhibiting DPDPE than deltorphin II, albeit to a lesser extent. However, all of the antagonists tested inhibited [^{35}S]GTPγS binding stimulated by DPDPE at a lower concentration than binding stimulated by deltorphin II (Fig. 1, Table 2). These results follow the receptor binding data, as all ligands tested had slightly higher affinities for the [^{3}H]DPDPE binding site compared to the [^{3}H]deltorphin II binding site (Table 1).

The cloned mouse δ-opioid receptor has been characterized as the δ_2-opioid receptor subtype based on the higher affinity of naltriben compared to BNTX for the [^{3}H]naltrindole binding site (Raynor et al., 1994). Our data with the cloned human δ-opioid receptor showed a similar trend, but another putative δ_1-opioid receptor-selective compound, DPDPE, had nearly equal affinity as the δ_2-opioid receptor-selective compounds, deltorphin II and NTII. Thus, only naltriben had significant higher affinity than the δ_1-opioid receptor-selective compounds. We caution, however, that this high affinity of naltriben is probably due to the compound it is derived from because its parent compound, naltrindole, also shows high affinity for all δ binding sites.

Our results clearly demonstrate that the ligands tested have no in vitro selectivity for the DPDPE or the deltorphin II binding sites. Yet, the existence of subtypes in vivo is still pharmacologically possible. Only one human δ-opioid receptor gene has been cloned (Simonin et al., 1994), so it is highly unlikely that expression of two separate genes could account for the presence of subtypes in vivo. Yet, the alternative splicing of the δ-opioid receptor gene could be a possible explanation of subtypes. Antisense mapping studies found that supraspinal analgesia induced by DPDPE was blocked only by antisense probes targeted to exon 3, whereas deltorphin II-induced analagesia could be blocked by antisense probes targeted to all three exons (Rossi et al., 1997). However, two other groups found no evidence for splice variants using the reverse transcriptase-polymerase chain reaction (Allouche et al., 2000; Simonin et al., 1994). The recent discovery of δ-opioid receptor homodimerization (Cvejic and Devi, 1997) and heterodimerization (Gomes et al., 2000) and -oligomerization (George et al., 2000) provides a possible explanation for the existence of subtypes in vivo. Particularly intriguing is that treatment with deltorphin II, but not DPDPE, increased [^{3}H]DAMGO binding in human embryonic kidney (HEK-293) cells coexpressing both the μ- and δ-opioid receptors (Gomes et al., 2000). These data led the authors to propose that the μ–δ heterodimer may represent the δ_2-opioid receptor subtype. However, homodimers and heterodimers have not yet been demonstrated to exist in vivo. Another possible explanation for receptor subtypes is differential G-protein activation by different ligands. Researchers showed that the δ-opioid receptor can couple to several G-protein α subunits including, $G_{i\alpha1}$, $G_{i\alpha3}$, $G_{o\alpha}$, $G_{q\alpha}$, and $G_{z\alpha}$ (Law and Resine, 1997). Antisense studies indicated that administration of antisense oligodeoxynucleotides to $G_{o1\alpha}$ can block DPDPE-induced antinociception in mice, but not antinociception induced by deltorphin II (Sanchez-Blazquez and Garzon, 1998). Conversely, antisense oligodeoxynucleotides to the pertussis toxin-insensitive $G_{q\alpha}$ blocked deltorphin II-induced antinociception, but not antinociception produced by DPDPE (Sanchez-Blazquez and Garzon, 1998). Yet, in vitro, pertussis toxin pretreatment abolishes all δ-opioid receptor-mediated responses in C_6 glioma cells (Clark et al., 1997). Probably, the most likely explanation of subtypes in vivo is

the differential accessibility of the ligands to different receptor populations. The putative δ_2-opioid receptor-selective ligands are accessible to a greater number of δ-opioid receptors. [^{3}H]Naltriben has a four-fold higher brain penetration than [^{3}H]BNTX (Lever et al., 1996), and deltorphin II has a very high blood brain permeability coefficient and a high in vitro stability (Thomas et al., 1997).

In conclusion, the putative δ_1- and δ_2-opioid receptor-selective ligands can bind and potently activate the δ-opioid receptor. Additionally, δ_1- and δ_2-opioid receptor-selective antagonists can inhibit both δ_1- and δ_2-opioid receptor-induced responses. Thus, the cloned human δ-opioid receptor cannot be classified as being either the δ_1- or the δ_2-opioid receptor subtype. Therefore, our evidence suggests that the findings in vivo are possibly due to the differential effects of these compounds in vivo and not because of the molecular existence of δ-opioid receptor subtypes.

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