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Effects of chronic administration of 7-benzylidene-7-dehydronaltrexone and naltriben on the antinociceptive actions of δ_1- and δ_2-opioid receptor agonists

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Abstract

The effects of chronic administration of 7-benzylidene-7-dehydronaltrexone, a δ_1-opioid receptor antagonist and naltriben, a δ_2-opioid receptor antagonist, on the antinociceptive responses to [D-Pen^2, D-Pen^5]enkephalin and [D-Ala^2, Glu^4]deltorphin II, δ_1- and δ_2-opioid receptor agonists, respectively, were determined in the mouse. Female B6C3F1 mice were given 7-benzylidene-7-dehydronaltrexone (3 mg/kg/day), naltriben (1 mg/kg/day) or the vehicle by subcutaneously implanted Alzet osmotic minipumps for 7 days. Both [D-Pen^2, D-Pen^5]enkephalin and [D-Ala^2, Glu^4]deltorphin II administered intracerebroventricularly (i.c.v.) produced antinociceptive as measured by the tail-flick test with ED_{50} values of 6.76 and 6.68 μg/mouse, respectively. Chronic administration of 7-benzylidene-7-dehydronaltrexone lowered the ED_{50} of [D-Pen^2, D-Pen^5]enkephalin but not of [D-Ala^2, Glu^4]deltorphin II. Chronic administration of naltriben lowered the ED_{50} of [D-Ala^2, Glu^4]deltorphin II but had no effect on the ED_{50} of [D-Pen^2, D-Pen^5]enkephalin. The binding of [^{3}H][D-Pen^2, D-Pen^5]enkephalin to whole brain membranes of chronic 7-benzylidene-7-dehydronaltrexone-treated mice did not differ from chronic vehicle-treated mice. On the other hand, chronic administration of naltriben resulted in slight but reproducible elevation in the B_{max} value of [^{3}H][D-Pen^2, D-Pen^5]enkephalin to bind to whole brain membranes in comparison to vehicle-injected controls. The results suggest that chronic treatment with δ_1- and δ_2-opioid receptor antagonist cause behavioral supersensitivity to their agonists, respectively, and provides further evidence for the existence of δ-opioid receptor subtypes.

Keywords: δ-Opioid receptor, agonist, antagonist; δ_1-Opioid receptor; δ_2-Opioid receptor; Antinociception; [^{3}H][D-Pen^2,D-Pen^5]Enkephalin

1. Introduction

Opioid drugs produce their pharmacological actions by interacting with three types of receptors, viz. μ, δ and κ (Bhargava, 1994). The possible existence of subtypes of these receptors has been suggested. Thus, μ- and κ-opioid receptors have been further classified into μ_1 and μ_2, and κ_1, κ_2 and κ_3 based on behavioral and biochemical differences in the activity of related compounds. Recently, two subtypes of δ-opioid receptors have been identified, and labeled as δ_1 and δ_2. The prototypical agonists at these receptors are [D-Pen^2, D-Pen^5]enkephalin and [D-Ala^2, Glu^4]deltorphin II, respectively (Mattia et al., 1991). Such classification is based on the fact that naltriben, a highly selective δ_2-opioid receptor antagonist, is more effective in antagonizing the antinociceptive action of [D-Ser^2, Leu^5, Thr^6]enkephalin than that of [D-Pen^2, D-Pen^5]enkephalin in mice (Sofuoglu et al., 1991). Lack of cross-tolerance between [D-Ser^2, Leu^5, Thr^6]enkephalin and [D-Pen^2, D-Pen^5]enkephalin or between [D-Pen^2, D-Pen^5]enkephalin and [D-Ala^2, Glu^4]deltorphin II provided additional evidence for δ-opioid receptor heterogeneity (Mattia et al., 1991). Differential antagonism of δ-opioid receptor agonists of [D-Pen^2, D-Pen^5]enkephalin and [D-Ala^2, Glu^4]deltorphin II-induced antinociceptive by the irreversible δ-opioid receptor antagonists, [D-Ala^2, Leu^5, Cys^6]enkephalin and naltrindole-5$'$-isothiocyanate also indicated the possibility of δ-opioid receptor subtypes (Jiang et al., 1991).

Recent studies in our laboratory have provided immunological evidence for the existence of subtypes of δ-opioid receptors. In general, [D-Pen^2, D-Pen^5]enkephalin, a δ_1-opioid receptor agonist was found to have greater im-

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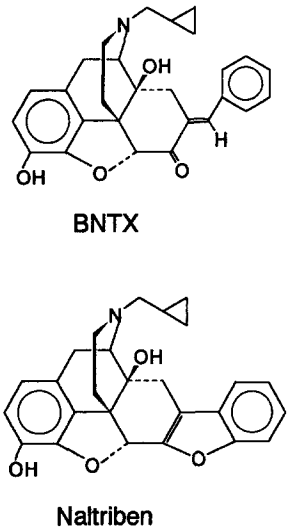

Fig. 1. Chemical structures of 7-benzylidene-7-dehydronaltrexone (BNTX) and naltriben.

munostimulant activity than [D-Ser^2, Leu^5, Thr^6]enkephalin or other δ_2-opioid receptor agonists (Bhargava et al., 1995a). On the other hand, 7-benzylidene-7-dehydronaltrexone, a highly selective δ_1-opioid receptor antagonist produced immunosuppression whereas naltriben was devoid of any effect on cellular immune function (House et al., 1995).

Since 7-benzylidene-7-dehydronaltrexone and naltriben have been designated as highly selective δ_1- and δ_2-opioid receptor antagonists, respectively, we tested the hypothesis that chronic blockade of δ_1- and δ_2-opioid receptors should up-regulate their own receptors. For this purpose, the effects of chronic administration of 7-benzylidene-7-dehydronaltrexone and naltriben (Fig. 1), by using osmotic minipumps, on the antinociceptive responses to [D-Pen^2, D-Pen^5]enkephalin and [D-Ala^2, Glu^4]deltorphin II and the binding of [^{3}H][D-Pen^2, D-Pen^5]enkephalin to brain membranes were determined in mice.

2. Materials and methods

2.1. Animals

Female, 6–8-week-old $B_6C_3F_1$ (B57BL6XC3H) mice obtained from Charles River Laboratories (Wilmington, MA, USA) were acclimated to a room with controlled ambient temperature (23 ± 1°C), humidity (50 ± 10%) and a 12-h dark-light cycle (light 06:00–18:00 h). The animals were housed under these conditions for at least 4 days before being used. The animals were given food and water ad libitum.

2.2. Drugs

7-Benzylidene-7-dehydronaltrexone HCl, naltriben methane sulfonate, [D-Pen^2, D-Pen^5]enkephalin and [D-Ala^2, Glu^4]deltorphin II were supplied by the Research Technology Branch, National Institute on Drug Abuse (Rockville, MD, USA) through the courtesy of Mr. Kevin Gormley. 7-Benzylidene-7-dehydronaltrexone and naltriben were dissolved in 10% dimethylsulfoxide in saline and administered subcutaneously to mice via Alzet osmotic minipumps (Model 1007 D) which delivered the drug solution at a rate of 0.5 μl/h for 7 days. The daily dose of 7-benzylidene-7-dehydronaltrexone and naltriben were 3 and 1 mg/kg, respectively. Mice which served as controls were implanted with osmotic minipumps filled with the vehicle. The osmotic minipumps were removed 7 days after their implantation. The implantation and removal of the pumps were done under light ether anesthesia.

[D-Pen^2, D-Pen^5]enkephalin and [D-Ala^2, Glu^4]deltorphin II were dissolved in water and 10% dimethylsulfoxide in water solution, respectively, and were injected i.c.v. under light ether anesthesia in a volume of 5 μl/mouse according to the method of Haley and McCormick (1957) as described previously (Bhargava and Zhao, 1996; Zhao and Bhargava, 1996). The coordinates for the injection site were 2 mm lateral and caudal to bregma at a depth of 3 mm by using a 10-μl Hamilton syringe with a 27-gauge needle.

2.3. Measurement of the antinociceptive response

The antinociceptive response to [D-Pen^2, D-Pen^5]enkephalin and [D-Ala^2, Glu^4]deltorphin II was measured by the tail-flick test as described previously (D'Amour and Smith, 1941; Bhargava and Thorat, 1994; Bhargava et al., 1995b). At the beginning of the study, the light intensity in the tail-flick apparatus was adjusted such that the mean basal latencies for the tail-flick response were approximately 2 s. To minimize tail skin tissue damage, the cut-off time was set at 10 s. The tail-flick latencies were determined before and 15 min after the i.c.v. injection of an appropriate dose of [D-Pen^2, D-Pen^5]enkephalin or [D-Ala^2, Glu^4]deltorphin II. A delay of 3 times the tail-flick latency over the basal value after i.c.v. injection of the drug was considered to be a positive response. Percentage of mice exhibiting antinociceptive response was calculated for each dose of the drug. Ten mice were used for each dose of the drug. Dose-response curves were constructed and the ED_{50} values and their confidence limits were determined by the method of Litchfield and Wilcoxon (1949).

2.4. Effects of chronic administration of 7-benzylidene-7-dehydronaltrexone and naltriben on the antinociceptive response to [D-Pen^2, D-Pen^5]enkephalin and [D-Ala^2, Glu^4]deltorphin II in mice

Seven days after the continuous administration of 7-benzylidene-7-dehydronaltrexone, naltriben or the vehicle, the osmotic minipumps were removed under light ether

anesthesia. The antinociceptive response to [D-Pen^2, D-Pen^5]enkephalin and [D-Ala^2, Glu^4]deltorphin II were determined 6 h after the removal of the osmotic minipumps. The ED_{50} values and their 95% confidence limits of the antinociceptive response to [D-Pen^2, D-Pen^5]enkephalin and [D-Ala^2, Glu^4]deltorphin II in mice injected with the vehicle, 7-benzylidene-7-dehydronaltrexone or naltriben were determined as described above.

2.5. Determination of binding of [^{3}H][D-Pen^2, D-Pen^5]enkephalin to brain membranes

2.5.1. Membrane preparation

Mice were sacrificed and the brain quickly excised into an ice-cold Petri dish. The cerebellum was removed and the remainder of the brain was homogenized in 60 vols. of ice-cold Tris-HCl buffer (0.05 M, pH 7.4) using a Brinkman polytron homogenizer (setting 5 for 20 s). The homogenate was centrifuged at 49 000 × g for 15 min in a refrigerated Sorvall RC-5B centrifuge. The process was repeated once more. After the second centrifugation, the pellet was stored at −80°C. For the binding assay, the pellet was suspended in 25 vols. of Tris-HCl buffer by homogenizing for 15 s as described above.

2.5.2. Binding assays

The binding of [^{3}H][D-Pen^2, D-Pen^5]enkephalin was performed as described previously (Bhargava et al., 1991; Magnan et al., 1982). Binding was carried out in a total volume of 0.5 ml which contained 0.2 ml of homogenate (450–500 μg protein) and 0.05 M Tris-HCl buffer. In saturation experiments, the [^{3}H][D-Pen^2, D-Pen^5]enkephalin concentration range was 1.0–40.0 nM. All binding assays were done in duplicate at 37°C for 60 min. Binding was terminated by rapidly filtering the contents of the incubation tubes through Whatman GF/B glass fiber filter under reduced pressure using a Brandell cell harvester (model M-24R). The filters were washed twice with 5 ml of the ice-cold 0.05 M Tris-HCl buffer. The filters were transferred to liquid scintillation vials containing 5 ml of SCINT-AXF scintillation fluid (Packard Instruments, Meriden, CT, USA). After an overnight equilibration period, the radioactivity in the samples was determined using a Packard liquid scintillation counter (model 4640) with a 54% counting efficiency. Specific binding was defined as the difference in binding observed in the absence and presence of 3.5 μM unlabeled [D-Pen^2, D-Pen^5]enkephalin. The concentration of protein in the samples was determined by employing the method of Lowry et al. (1951).

Receptor density (B_{max}) and apparent dissociation constant (K_d) for the binding of [^{3}H][D-Pen^2, D-Pen^5]enkephalin to brain membranes were determined from the saturation curves and Scatchard plots using the LIGAND program (Munson and Rodbard, 1980). The results were expressed as mean ± S.E.M. Six to 8 mice were used to determine the binding constants.

2.5.3. Effect of chronic administration of 7-benzylidene-7-dehydronaltrexone and naltriben on the binding of [^{3}H][D-Pen^2, D-Pen^5]enkephalin to mouse brain membranes

Seven days after the continuous administration on 7-benzylidene-7-dehydronaltrexone and naltriben or the vehicle, the osmotic minipumps were removed under light ether anesthesia. Six hours later, mice were decapitated and the brains quickly removed for the binding of [^{3}H][D-Pen^2, D-Pen^5]enkephalin as described above. The B_{max} and K_d values were determined in brains from all the treatment groups. The differences in the binding constants of [^{3}H][D-Pen^2, D-Pen^5]enkephalin in vehicle- and drug-treated mice was determined by Student's t-test. A value of $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Effect of chronic administration of 7-benzylidene-7-dehydronaltrexone and naltriben on the antinociceptive response to [D-Pen^2, D-Pen^5]enkephalin

Chronic administration of 7-benzylidene-7-dehydronaltrexone enhanced the antinociceptive response to [D-Pen^2, D-Pen^5]enkephalin as evidenced by a leftward shift in its dose-response curve in 7-benzylidene-7-dehydronaltrexone-treated mice in comparison to vehicle-injected mice. The ED_{50} value of [D-Pen^2, D-Pen^5]enkephalin in vehicle-injected mice was 6.76 μg/mouse which was decreased by almost 50% to 3.41 μg/mouse in 7-benzylidene-7-dehydronaltrexone-treated mice (Table 1). On the other hand, the antinociceptive response to [D-Pen^2, D-Pen^5]enkephalin was not altered in mice treated chronically with naltriben (ED_{50} value 6.82 μg/mouse) when compared to vehicle-injected mice. The dose-response curves for [D-Pen^2, D-Pen^5]enkephalin in vehicle- and naltriben-treated mice did not differ and thus the ED_{50} values of [D-Pen^2, D-Pen^5]enkephalin also did not change (Table 1).

Table 1
Effect of chronic administration of 7-benzylidene-7-dehydronaltrexone (BNTX) and naltriben on the antinociceptive response to [D-Pen^2, D-Pen^5]enkephalin in $B_6C_3F_1$ mice

Treatment	ED_{50} of [D-Pen^2, D-Pen^5]enkephalin (μg/mouse)
Vehicle	6.76 (4.12–11.06) [a]
BNTX	3.41 (2.03–5.73) [a,b]
Naltriben	6.82 (4.05–11.47) [a]

Mice were administered vehicle, 7-benzylidene-7-dehydronaltrexone or naltriben by Alzet osmotic minipumps for 7 days as described in the text. The antinociceptive response to different doses of i.c.v. injected [D-Ala^2, Glu^4]deltorphin II was determined by the tail-flick test.

[a] 95% confidence limits.

[b] $P < 0.05$ vs. vehicle-treated group.

Table 2
Effect of chronic administration of 7-benzylidene-7-dehydronaltrexone (BNTX) and naltriben on the antinociceptive response to [D-Ala^2, Glu^4]deltorphin II in $B_6C_3F_1$ mice

Treatment	ED_{50} of [D-Ala^2, Glu^4]deltorphin II (μg/mouse)
Vehicle	6.68 (5.18–8.62) [a]
BNTX	6.67 (4.99–8.92) [a]
Naltriben	3.72 (2.48–5.58) [a,b]

Mice were administered vehicle, 7-benzylidene-7-dehydronaltrexone or naltriben by Alzet osmotic minipumps for 7 days as described in the text. The antinociceptive response to different doses of i.c.v. injected [D-Ala^2, Glu^4]deltorphin II was determined by the tail-flick test.
[a] 95% confidence limits.
[b] $P < 0.05$ vs. vehicle-injected controls.

Table 3
Effect of chronic administration of 7-benzylidene-7-dehydronaltrexone (BNTX) and naltriben on the binding of [^{3}H][D-Pen^2, D-Pen^5]enkephalin to brain membranes of $B_6C_3F_1$ mice

Treatment	[^{3}H][D-Pen^2, D-Pen^5]enkephalin binding constants (mean ± S.E.M. $n = 6–8$)	
	B_{max} (fmol/mg protein)	K_d (nM)
Vehicle	371 ± 13	6.74 ± 0.30
BNTX	379 ± 17	6.80 ± 0.60
Naltriben	412 ± 10 *	7.49 ± 0.68

The binding [^{3}H][D-Pen^2, D-Pen^5]enkephalin was carried out in a concentration of 1.0–40.0 nM. Specific binding was defined as the binding observed in the absence and presence of 3.5 μM unlabeled [D-Pen^2, D-Pen^5]enkephalin.
* $P < 0.05$ vs. vehicle-injected controls.

3.2. Effect of chronic administration of 7-benzylidene-7-dehydronaltrexone and naltriben on the antinociceptive response to [D-Ala^2, Glu^4]deltorphin II

Chronic administration of naltriben enhanced the antinociceptive response to [D-Ala^2, Glu^4]deltorphin II as evidenced by a leftward shift in its dose-response curve in naltriben-treated mice in comparison to vehicle-injected mice. The ED_{50} value of [D-Ala^2, Glu^4]deltorphin II in naltriben-treated mice was 3.72 μg/mouse, which was significantly lower than in mice treated chronically with vehicle (6.68 μg/mouse) (Table 2). The antinociceptive response to [D-Ala^2, Glu^4]deltorphin II, on the other hand, was not affected in mice treated chronically with 7-benzylidene-7-dehydronaltrexone (6.67 μg/mouse) when compared to vehicle-injected mice. The dose-response curves for [D-Ala^2, Glu^4]deltorphin II in vehicle and 7-benzylidene-7-dehydronaltrexone-treated overlapped; consequently the ED_{50} values for [D-Ala^2, Glu^4]deltorphin II in the two treatment groups were virtually identical (Table 2).

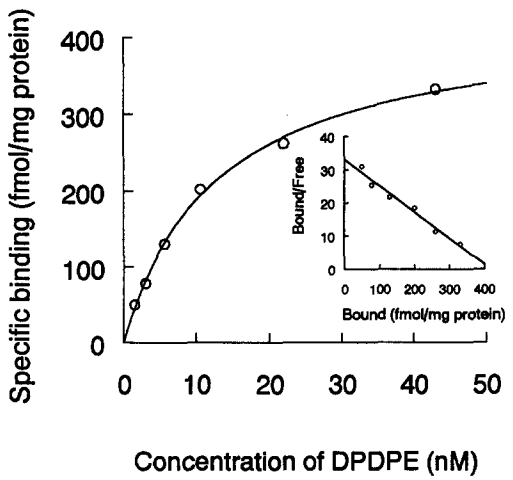

Fig. 2. A typical saturation curve and Scatchard plot (inset) for the binding of [^{3}H][D-Pen^2, D-Pen^5]enkephalin to mouse brain membranes.

3.3. Effect of chronic administration on 7-benzylidene-7-dehydronaltrexone and naltriben on the binding of [^{3}H][D-Pen^2, D-Pen^5]enkephalin to mouse brain membranes

[^{3}H][D-Pen^2, D-Pen^5]enkephalin bound to the mouse brain membranes at a single high-affinity site with a B_{max} value of 371 fmol/mg protein and a K_d value of 6.7 nM. A typical Scatchard plot of the binding of [^{3}H][D-Pen^2, D-Pen^5]enkephalin to brain membranes is shown in Fig. 2. As can be seen in Table 3, chronic administration of 7-benzylidene-7-dehydronaltrexone did not alter the binding constants of [^{3}H][D-Pen^2, D-Pen^5]enkephalin in the mouse brain. However, a small (10%) but significant increase in the B_{max} value of [^{3}H][D-Pen^2, D-Pen^5]enkephalin was observed in mice treated chronically with naltriben; K_d value was unaffected.

4. Discussion

The present studies clearly indicate that the chronic blockade of δ_1-opioid receptor by 7-benzylidene-7-dehydronaltrexone enhances the antinociceptive action of [D-Pen^2, D-Pen^5]enkephalin, a δ_1-opioid receptor agonist. However, such a treatment had no effect on the antinociceptive response to [D-Ala^2, Glu^4]deltorphin II, a highly selective δ_2-opioid receptor agonist. On the other hand, chronic blockade of the δ_2-opioid receptor by naltriben enhanced the antinociceptive action of [D-Ala^2, Glu^4]deltorphin II, but had no effect on the antinociceptive response to [D-Pen^2, D-Pen^5]enkephalin. These results provide evidence for the functional up-regulation of δ_1- and δ_2-opioid receptors as a result of their chronic blockade.

The results of the present studies further confirm the existence of δ-opioid receptors subtypes. Previous studies have also provided behavioral evidences for the existence of subtypes of δ-opioid receptors. Thus, naltriben was shown to antagonize the antinociceptive action of [D-Ser^2, Leu^5, Thr^6]enkephalin but not of DADLE and [D-Pen^2,

D-Pen^5]enkephalin (Sofuoglu et al., 1991). Additionally, a lack of cross-tolerance between [D-Pen^2, D-Pen^5]enkephalin and [D-Ala^2, Glu^4]deltorphin II has been demonstrated (Mattia et al., 1991). However, to date, little biochemical (binding) evidence has been provided for such a phenomenon.

To determine whether the enhanced responses to δ_1- and δ_2-opioid receptor agonists in 7-benzylidene-7-dehydronaltrexone- and naltriben-treated mice, respectively, were related to alterations in the δ-opioid receptor characteristics, the binding of [^{3}H][D-Pen^2, D-Pen^5]enkephalin to brain membranes was determined. It was clear from the present studies that B_{max} and K_d values of [^{3}H][D-Pen^2, D-Pen^5]enkephalin were unaffected by 7-benzylidene-7-dehydronaltrexone treatment. However, there was a 10% increase in B_{max} of [^{3}H][D-Pen^2, D-Pen^5]enkephalin in the brain of naltriben-treated mice over the controls but although the K_d values did not differ. This effect, although small was consistently reproducible. Thus, the enhanced antiniciceptive response to [D-Pen^2, D-Pen^5]enkephalin in mice treated chronically with 7-benzylidene-7-dehydronaltrexone can not be explained by the changes in the characteristics of δ-opioid receptors labeled with [^{3}H][D-Pen^2, D-Pen^5]enkephalin. Similarly, a 45% decrease in the ED_{50} value of [D-Ala^2, Glu^4]deltorphin II could not be accounted for by a 10% increase in the B_{max} value of [^{3}H][D-Pen^2, D-Pen^5]enkephalin in naltriben-treated mice. Since there is no cross-tolerance between [D-Pen^2, D-Pen^5]enkephalin and [D-Ala^2, Glu^4]deltorphin II, a 10% increase in the B_{max} of [^{3}H][D-Pen^2, D-Pen^5]enkephalin would not account for the enhanced response to [D-Ala^2, Glu^4]deltorphin II in naltriben-treated mice.

Although naltriben could distinguish between [D-Pen^2, D-Pen^5]enkephalin and [D-Ser^2, Leu^5, Thr^6]enkephalin behaviorally, it was shown to compete equally well against the binding of [^{3}H][D-Pen^2, D-Pen^5]enkephalin and [^{3}H][D-Ser^2, Leu^5, Thr^6]enkephalin in brain membranes (Sofuoglu et al., 1992). Thus, binding studies have not been able to distinguish between δ_1- and δ_2-opioid receptors.

In another study, chronic administration of naltrexone, a non-specific opioid receptor antagonist, was shown to shift the antinociceptive dose-response curve for [D-Ala^2, Glu^4]deltorphin II to the left, i.e. there was supersensitivity to the antinociceptive effect of [D-Ala^2, Glu^4]deltorphin II. However, the levels of mRNA for the δ-opioid receptors in the brain were unaffected (Jenab et al., 1995). Thus, the enhancement of δ-opioid receptor agonist induced antinociceptive response by chronic blockade of δ-opioid receptors does not appear to be due to changes in either the characteristics (B_{max} or K_d) or the synthesis (mRNA levels) of the δ-opioid receptors. Similarly, chronic i.c.v. administration of [D-Pen^2, D-Pen^5]enkephalin resulted in the development of tolerance to its antinociceptive action but the level of brain mRNA for δ-opioid receptors was unaffected (Kest et al., 1995).

Naltrindole is a non-selective δ-opioid receptor antagonist and has been reported to exhibit immunosuppressive activity (Arakawa et al., 1992, 1993). With the development of more selective δ_1- and δ_2-opioid receptor antagonists, we have recently begun to examine the immunomodulation by selective and non-selective δ-opioid receptor agonists and antagonists in vitro (House et al., 1995). The effects of naltrindole as well as of 7-benzylidene-7-dehydronaltrexone and naltriben on the cellular immune function were determined. In vitro exposure to 7-benzylidene-7-dehydronaltrexone resulted in an apparent dose-related suppression of B-cell proliferation, cytokine production by T-helper cells, and natural killer cell activity. Naltrindole was also immunosuppressive for all immune function parameters examined, although it was less active than 7-benzylidene-7-dehydronaltrexone. On the other hand, in vitro exposure to naltriben had no significant effect on any immune function examined (House et al., 1995). These studies clearly indicated that highly selective δ_1-opioid receptor antagonist is immunosuppressive whereas δ_2-opioid receptor antagonist is not, and provide additional functional evidence for the heterogeneity of δ-opioid receptors.

In summary, the present study provides further evidence for the presence of subtypes of δ-opioid receptors and further demonstrate that 7-benzylidene-7-dehydronaltrexone and naltriben are highly selective antagonists for the δ_1- and δ_2-opioid receptors, respectively.

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