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Effects of competitive and noncompetitive antagonists of the N-methyl-D-aspartate receptor on the analgesic action of δ_1- and δ_2-opioid receptor agonists in mice

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1 The effects of MK-801, a noncompetitive antagonist of the N-methyl-D-aspartate (NMDA) receptor and LY 235959, a competitive antagonist of the NMDA receptor on the analgesic actions of [D-Pen^2,D-Pen^5]enkephalin (DPDPE) and [D-Ala^2, Glu^4] deltorphin II (deltorphin II), the putative δ_1- and δ_2-opioid receptor agonists, respectively, were determined in the male Swiss-Webster mice.

2 Intracerebroventricular administration of DPDPE or deltorphin II produced analgesia. MK-801 administered intraperitoneally 10 min before the injection of DPDPE or deltorphin II, dose-dependently antagonized the analgesic actions of both drugs.

3 LY 235959 also dose-dependently antagonized the analgesic actions of DPDPE and deltorphin II.

4 The effects of MK-801 and LY 235959 on the binding of [^{3}H]-DPDPE to mouse brain membranes were also determined. Neither of the NMDA receptor antagonists had any effect on the binding of [^{3}H]-DPDPE.

5 It is concluded that competitive and noncompetitive antagonists of the NMDA receptor antagonize the analgesic action of δ_1- and δ_2-opioid receptor agonists and that such effects are not mediated via a direct interaction with brain δ-opioid receptors.

Keywords: δ-Opioid receptor agonists; analgesia; NMDA receptor antagonists; MK-801; LY 235959; [^{3}H]-[D-Pen^2,D-Pen^5]enkephalin ([^{3}H]-DPDPE) binding

Introduction

It is well established that opioid drugs produce their actions by interacting with at least three types of opioid receptor, namely μ, κ and δ. The prototypical agonists at these receptors are morphine or [D-Ala^2,MePhe^4,Gly-ol^5]enkephalin (DAMGO), U-50,488H or dynorphin and [D-Pen^2,D-Pen^5]enkephalin (DPDPE), respectively (Bhargava, 1994). In recent years, several studies have been undertaken to determine the potential role of excitatory amino acids in the analgesic responses to opioid drugs in mice and rats. However, contradictory results have been obtained. For example, in the rat dizocilpine (MK-801), a noncompetitive antagonist of the N-methyl-D-aspartate (NMDA) receptor was found to antagonize U-50,488H but not the morphine-induced antinociception (Kest *et al.*, 1992). On the other hand, acute and chronic administration of MK-801 was shown to enhance U-50,488H-induced analgesia in the rat. Furthermore, *in vitro* and *in vivo* treatment with MK-801 affects the brain and spinal cord κ-opioid receptors. *In vitro*, MK-801 inhibited the binding of [^{3}H]-ethylketocyclazocine (EKC) to rat brain and spinal cord membranes while chronic administration of MK-801 appeared to increase the κ-opioid receptor density in the brain (Bhargava *et al.*, 1995).

In the mouse, however, MK-801 has been shown to antagonize the analgesic action of morphine (Lipa & Kavaliers, 1990). The antagonism of the analgesic action of morphine by MK-801 did not appear to involve brain opioid receptors, since MK-801 exhibited poor affinity for the binding of [^{3}H]-naloxone, an antagonist with predominant action at the μ-opioid receptor (Lutfy *et al.*, 1993).

Little is known about the interaction of excitatory amino acid receptors and δ-opioid receptor agonists. Both behavioural and biochemical evidence have been presented for the existence of subtypes of δ-opioid receptors. Thus, DPDPE and deltorphin II on intracerebroventricular (i.c.v.) administration in mice produced tolerance to their analgesic actions. However, mice tolerant to DPDPE did not show cross-tolerance to deltorphin II (Mattia *et al.*, 1991). Furthermore, two antagonists of the δ-opioid receptors, naltrindole (NTI) and naltriben (NTB) differentially affected the analgesic responses to i.c.v. administered [D-Ser^2,Leu^5,Thr^6]enkephalin (DSLET) and DPDPE (Sofuoglu *et al.*, 1991). Similarly, [D-Ala^2, Leu^5, Cys^6]enkephalin (DALCE) and naltrindole 5′-isothiocyanate (5′-NTII) produced differential antagonism of i.c.v. administered DPDPE and deltorphin II in mice (Jiang *et al.*, 1991).

Biochemical evidence for the heterogeneity of δ-opioid receptor binding sites has been provided by the interaction of various δ-opioid receptor agonists with the binding of [^{3}H]-DPDPE, [^{3}H]-DSLET and [^{3}H]-[D-Ala^2,D-Leu^5]enkephalin (DADLE) to mouse brain homogenates (Sofuoglu *et al.*, 1992). Based on behavioural and biochemical studies, DPDPE and deltorphin II have been proposed as highly selective putative δ_1- and δ_2-opioid receptor agonists (Mattia *et al.*, 1991).

The present studies were undertaken to determine whether competitive and noncompetitive antagonists of the NMDA receptors have any effect on the δ_1- and δ_2-opioid receptor agonists-induced analgesia. For this purpose, DPDPE and deltorphin II were selected as the δ_1- and δ_2-opioid receptor agonists. The competitive and noncompetitive antagonists of the NMDA receptor used were (−)-6-phosphonomethyl-decahydro-isoquinoline-3-carboxylic acid (LY 235959) and (+)-5-methyl-10,11-dihydro-5H-dibenzyl[a,d] cyclohepten-5,10-imine hydrogen maleate (dizocilpine or MK-801).

Methods

Animals

Male Swiss Webster mice weighing 25–30 g (Taconic Animal Co., Germantown, NY) were housed 5 to a cage in a room

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with controlled temperature (23±1°C), humidity (50±10%) and light (06 h 00 min–20 h 00 min) for at least 4 days before being used. Food and water were made available *ad libitum*.

Measurement of the analgesic response

The analgesic response to DPDPE and deltorphin II was measured by the tail-flick test as described previously (Bhargava & Matwyshyn, 1993; Bhargava, 1995; Bhargava *et al.*, 1995; D'Amour & Smith, 1941). 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 an i.c.v. injection of DPDPE (10 μg per mouse) or deltorphin II (10 μg per mouse). The basal tail-flick latencies were subtracted from the effect induced by the drug for each mouse; % maximum possible effect (%MPE) was calculated from the following formula:

$$\%MPE = [(\text{test latency} - \text{control latency})/(10 - \text{control latency})] \times 100$$

Ten mice were used for each treatment group and the data are expressed as mean % MPE±s.e.mean.

Effects of MK-801 and LY 235959 on the analgesic actions of DPDPE and deltorphin II in mice

To determine the effects of MK-801 and LY 235959 on the analgesic actions of DPDPE and deltorphin II, the NMDA receptor antagonists were injected i.p. in appropriate doses 10 min before the injections of DPDPE or deltorphin II. The doses of MK-801 used were 0.08, 0.12 and 0.16 mg kg^{-1} whereas those of LY23959 were 1.0, 2.0 and 4.0 mg kg^{-1} respectively. The tail-flick reaction times were determined before and 15 min after DPDPE or deltorphin II injection. %MPE values were calculated for each mouse as described above and expressed as mean %MPE±s.e.mean. Ten mice were used for each treatment group. Data were analysed by nonparametric Kruskal-Wallis one-way analysis of variance by ranks followed by the Dunnett's test. A value of $P<0.05$ was considered statistically significant.

Determination of binding of [^{3}H]-DPDPE to brain membranes

Membrane preparation Mice were killed and the brain quickly excised in an ice-cold Petri dish. The cerebellum was removed and the remainder of the brain was homogenized in 60 volumes of ice-cold Tris-HCl buffer (0.05 M, pH 7.4) with 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 volumes of Tris-HCl buffer by homogenizing for 15 s as described above.

Binding assays The binding of [^{3}H]-DPDPE 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]-DPDPE 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 fibre filter under reduced pressure with 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, U.S.A.). After an overnight equilibration period, the radioactivity in the samples was determined by 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 5.0 μM unlabelled DPDPE. 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]-DPDPE to brain membranes were determined from the saturation curves and Scatchard plots by use of the LIGAND program (Munson & Rodbard, 1980). Six mice were used to determine the binding constants. The results are expressed as mean value±s.e.mean.

Effects of MK-801 and LY 235959 on the binding of [^{3}H]-DPDPE to mouse brain membranes

The effects of MK-801 or LY 235959 (10^{-10}–10^{-4} M) on the binding of [^{3}H]-DPDPE 7 nM were determined in the mouse brain homogenate. Specific binding of [^{3}H]-DPDPE was determined as described above. Three mice were used for these studies.

Chemicals

MK-801 was purchased from Research Biochemical Inc., Natick, MA. LY 235959 was supplied generously by Eli Lilly and Co., Indianapolis, IN, through the courtesy of Dr Dennis M. Zimmermann. DPDPE, deltorphin II and [^{3}H]-DPDPE (specific activity 18 Ci $mmol^{-1}$) were obtained from the Research Technology Branch, National Institute on Drug Abuse, Rockville, MD, through the courtesy of Mr Kevin Gormley. MK-801 and LY 235959 were dissolved in physiological saline and injected intraperitoneally (i.p.) in a volume 10 ml kg^{-1} body weight. DPDPE was dissolved in water and deltophin II in 10% DMSO in water and injected i.c.v. in a volume of 5 μl per mouse according to the method of Haley & McCormick (1957).

Results

Effects of MK-801 on the analgesic response to DPDPE and deltophin II in mice

MK-801 attenuated the analgesic response to both DPDPE and deltorphin II in mice in a dose-related fashion. Kruskal-Wallis test revealed a highly significant interaction [H=14.3 (A); H=15.4 (B), $\chi^2_{0.005(3)}=12.8$, $P<0.005$]. As shown in Figure 1a, the decrease in analgesic effect of 10 μg per mouse of DPDPE amounted to 19, 39 and 76% at 0.08, 0.12 and 0.16 mg kg^{-1} doses, respectively, when compared to vehicle injected controls. The inhibition of the analgesic effect of deltorphin II (10 μg per mouse) by MK-801 at doses of 0.08, 0.12 and 0.16 mg kg^{-1} amounted to 30, 40 and 78%, respectively in comparison to vehicle-injected controls (Figure 1b).

Effects of LY 235959 on the analgesic action of DPDPE and deltorphin II in mice

LY 235959 also attenuated the analgesic response to DPDPE and deltorphin II in mice in a dose-dependent manner. A highly significant interaction was revealed by Kruskal-Wallis test [H=20.3 (A), H=69.6 (B), $\chi^2_{0.005\ (3)}=12.8$, $P<0.005$]. As can be seen in Figure 2a, LY 235959 inhibited the analgesic action of DPDPE, 10 μg per mouse, by 12, 55 and 92% at doses of 1.0, 2.0 and 4.0 mg kg^{-1}, respectively, when compared to the vehicle injected controls. The effect of LY 235959 on the analgesic response to deltorphin II is shown in Figure 2b. LY 235959 decreased the analgesic response to deltorphin II, 10 μg per mouse, by 26, 52 and 85%, at doses of 1.0, 2.0 and 4.0 mg kg^{-1}, respectively, when compared to vehicle injected controls.

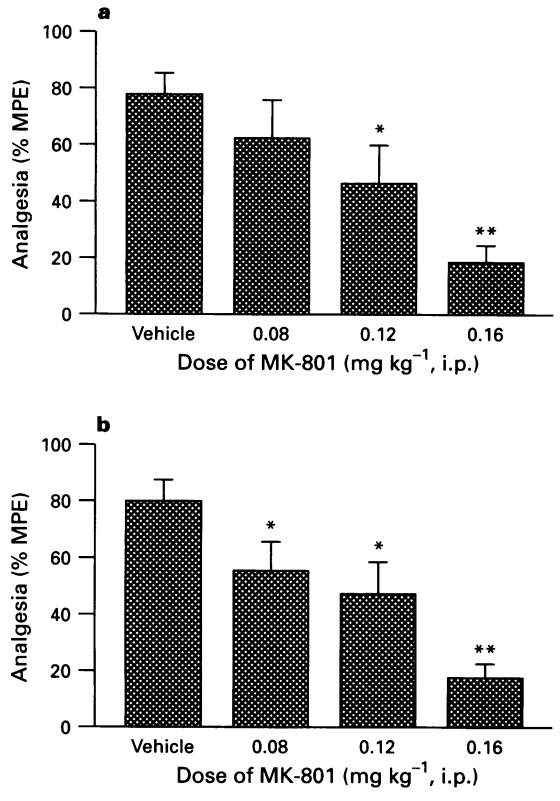

Figure 1 Effect of MK-801 on the analgesic response to DPDPE (a) or deltorphin II (b). Various doses of MK-801 were injected intraperitoneally 10 min before the intracerebroventricular injection of DPDPE or deltorphin II (10 μg per mouse). The analgesic response was measured by the tail-flick test as described in the text. Means ± s.e.mean ($n = 10$) are shown. *$P < 0.05$; **$P < 0.01$ vs. vehicle injected controls.

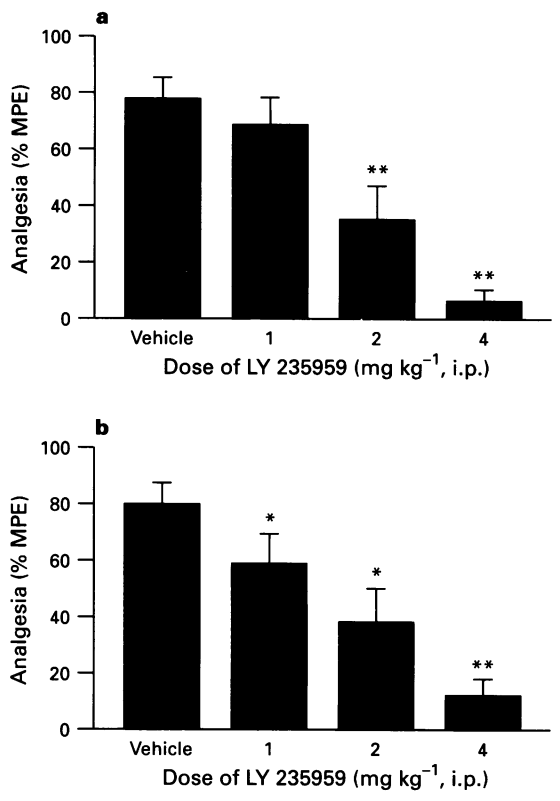

Figure 2 Effect of LY 235959 on the analgesic response to DPDPE (a) or deltorphin II (b). Various doses of LY 235959 were injected intraperitoneally 10 min before the intracerebroventricular injection of DPDPE or deltorphin II (10 μg per mouse). The analgesic response was measured by the tail-flick test as described in the text. Means ± s.e.mean ($n = 10$) are shown. *$P < 0.05$; **$P < 0.01$ vs. vehicle injected controls.

Effects of MK-801 and LY 235959 on the binding of [^{3}H]-DPDPE to mouse brain membranes

[^{3}H]-DPDPE bound to mouse brain membranes at a single high affinity site with a B_{max} of 395 ± 15 fmol mg^{-1} protein and a K_d of 7.20 ± 0.62 nM. The effects of MK-801 and LY 235959 on the binding of [^{3}H]-DPDPE, 7 nM, are shown in Table 1. Both compounds failed to show any significant effect on the binding of [^{3}H]-DPDPE to mouse brain membranes even at a concentration of 10^{-4} M.

Table 1 Effect of MK-801 and LY 235959 on the binding of [^{3}H]-DPDPE to mouse brain homogenates[a]

Drug concentration (−log M)	*Specific binding of [^{3}H]-DPDPE (% control)*[b] *MK-801*	*LY 235959*
10	91.1 ± 8.0	88.8 ± 1.5
9	87.3 ± 5.2	91.7 ± 1.5
8	90.0 ± 1.5	89.4 ± 1.4
7.5	91.6 ± 2.1	88.9 ± 1.4
7	94.4 ± 3.3	90.3 ± 0.3
6.5	88.8 ± 2.4	94.0 ± 3.6
6	100.3 ± 3.3	101.5 ± 1.0
5	90.6 ± 4.6	88.8 ± 4.9
4	73.5 ± 1.1	94.0 ± 4.3

[a]The concentration of [^{3}H]-DPDPE used was 7 nM. [b]Values represent mean ± s.e.mean ($n = 3$).

Discussion

The present studies demonstrate that both competitive and noncompetitive antagonists of the NMDA receptor dose-dependently antagonize the analgesia induced by DPDPE and deltorphin II in mice. The competitive antagonists like LY 235959 compete with the glutamate site whereas non-competitive antagonists like MK-801 block the calcium channel. It is interesting to note that the potency of MK-801 in antagonizing DPDPE and deltorphin II induced analgesia did not differ. Similar effects were observed with LY 235959. Thus, the NMDA receptor antagonist cannot be used to distinguish between the δ_1- and δ_2-opioid receptor agonists.

The mechanism by which the NMDA receptor antagonist inhibited the analgesic response to the δ-opioid receptor agonists is not clear. Our preliminary studies showed that the binding of [^{3}H]-DPDPE, a highly specific δ-opioid receptor agonist, to mouse brain membranes was unaffected by both MK-801 and LY 235959 even up to a concentration of 10^{-4} M. Thus, it does not appear that the antagonism of the analgesic actions of DPDPE and deltorphin II by NMDA receptor antagonists is mediated via a direct interaction with the central δ-opioid receptors.

Attempts have also been made to study the interactions of NMDA receptor antagonists with other types of opioid receptors. In the mouse, MK-801 was found to inhibit the binding of [^{3}H]-naloxone, a nonspecific opioid receptor antagonist with an IC_{50} value of 34 μM and antagonize the analgesic action of morphine (Lutfy *et al.*, 1993). On the other hand, MK-801 enhanced the analgesic action of U-50,488H, a κ-opioid receptor agonist in the rat and displaced the binding of [^{3}H]-ethylketocyclazocine to brain and spinal cord membranes with IC_{50} values of 9.8 and 1.4 μM, respectively

(Bhargava *et al.*, 1995). In another study, the IC_{50} values of LY 274614, the racemic form of LY 235959 in μ_1, μ_2, δ, κ_1 and κ_3 ligand binding assays were shown be greater than 10 μM (Tiseo *et al.*, 1994).

Several neurotransmitters are apparently involved in the modulation of pain processes. Opioid drugs can produce analgesia by inhibiting nociceptive input at supraspinal and spinal sites (Yaksh, 1984). These sites may be affected directly or indirectly by non-opioid agents in either an additive or antagonistic manner involving excitatory amino acids (EEAS) (Watkins & Evans, 1981; Wilcox, 1991). EEAS such as glutamate are present in the superficial dorsal horn C fibre central terminals along with substance P (Greenmayre *et al.*, 1984). Glutamate also activates superficial dorsal horn neurones in the spinal cord (Schneider & Perl, 1988). NMDA has been implicated in the 'wind-up' phenomenon, which causes central hyperalgesia, and this can be reversed by NMDA receptor antagonists (Goodchild, 1993). Additionally, spinal administration of NMDA receptor agonists results in hyperalgesia in the hot-plate and tail-flick assay in mice and rats (Aanonsen & Wilcox, 1987; Raigorodsky & Ucra, 1987). Thus, evidence has been presented on the possible involvement of EEAS in central nociceptive transmission.

Studies have also been carried out to determine the interaction of NMDA receptor systems and opioid drugs. The NMDA antagonist, MK-801, injected directly into the periaqueductal gray (PAG) matter antagonized morphine and NMDA antinociception suggesting that morphine produces analgesia in the PAG by disinhibition of neurones that contain NMDA receptors (Jacquet, 1988). Morphine antinociception, but not NMDA-induced antinociception, was also antagonized by the NMDA receptor antagonist, MK-801, injected into the nucleus raphe magnus (Van Praag & Frenk, 1990). Conflicting results have been obtained on the effects of acute peripheral administration of MK-801 on morphine antinociception. Whereas, in the rat, MK-801 does not appear to antagonize morphine antinociception (Marek *et al.*, 1991; Trujilo & Akil, 1991), it has been shown to antagonize it in the mouse (Lipa & Kavaliers, 1990; Lutfy *et al.*, 1993). In our studies, neither MK-801 nor LY235959 injected peripherally altered the basal tail-flick latency and thus did not produce analgesia or hyperalgesia, but both compounds antagonized the antinociception induced by δ_1- and δ_2-opioid receptor agonists in mice.

Previous studies from this laboratory have demonstrated that in the rat, MK-801 given acutely enhances the analgesic response of U-50,488H, a κ-opioid receptor agonist, to brain and spinal cord membranes with IC_{50} values of 10.0 μM and 1.4 μM, respectively (Bhargava *et al.*, 1995). It can be concluded that MK-801 may be acting as an agonist of the κ-opioid receptor. Furthermore, MK-801 was more potent at the spinal κ-opioid receptors than the supraspinal κ-opioid receptors. Thus, it is possible, although needs to be proven, that the antagonism of δ-opioid receptor agonists by MK-801 may involve κ-opioid receptors and/or the endogenous dynorphin system.

In summary, the present results demonstrate, for the first time, that noncompetitive and competitive antagonists of the NMDA receptor antagonize the analgesic actions of both the δ_1- and δ_2-opioid receptor agonist - induced analgesia in mice and that such effects are not mediated via the central δ-opioid receptors.

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