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Modulation of μ-mediated antinociception by δ agonists: characterization with antagonists

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The functional interactions between supraspinal μ and δ receptors were characterized in the mouse using μ receptor-selective antagonists. The effects of pretreatment with the μ opioid antagonists, β-funaltrexamine (β-FNA) and naloxonazine on the modulation of morphine antinociception by the δ agonists [D-Pen^2,D-Pen^5]enkephalin (DPDPE) and [D-Ala^2,Met^5]enkephalinamide (DAMA) were studied. When co-administered in the same i.c.v. injection, a sub-antinociceptive dose of DPDPE consistently and significantly increased the antinociceptive potency of morphine in control animals, while a sub-effective dose of DAMA decreased morphine antinociception; both the respective increase and the decrease of morphine potency by DPDPE and DAMA had been previously shown to be blocked by ICI 174,864, a δ antagonist. Pretreatment of mice with the non-equilibrium μ antagonist β-FNA 4 h prior to testing, a pretreatment which had no effect on i.c.v. DPDPE or DAMA antinociception, prevented the modulation of morphine antinociception by both DPDPE and DAMA. Pretreatment with the long acting μ_1 antagonist naloxonazine, 24 h prior to testing, failed to affect the modulation of morphine antinociception by either DPDPE or DAMA; such a pretreatment had no effect on the antinociceptive effects of DPDPE or DAMA when given alone. These results provide further support for the concept of a functionally coupled μ-δ receptor complex which is sensitive to antagonism by β-FNA, but not naloxonazine, and support the notion that subtypes of opioid μ and δ (i.e. complexed and non-complexed) receptors may exist.

Opioid antinociception; μ Opioid receptors; δ Opioid receptors; β-Funaltrexamine (β-FNA); Naloxonazine; (Intracerebroventricular, Mouse)

1. Introduction

The suggestion of multiple opioid receptor subtypes has been supported by a great deal of evidence obtained in vitro (Lord et al., 1977; Gioannini et al., 1985; Cho et al., 1986) and in vivo (Martin et al., 1976). Nevertheless, the correlation of opioid-induced effects with specific receptor subtypes has been difficult and continues to be the focus of a great deal of research. Of the effects studied, opioid-induced antinociception remains at the forefront of interest and relevance. Recent investigations, which used heat as the noxious stimulus, have demonstrated that supraspinal opioid-induced antinociception in mice can be mediated by both δ (Porreca et al., 1984; Heyman et al., 1987; Mathiasen et al., 1987; Porreca et al., 1987; Takemori and Portoghese, 1987) and μ opioid receptors.

In addition to the direct role played by the δ receptor in production of antinociception in this species, data also exist which suggest that [Leu^5]- and [Met^5]enkephalin, endogenous δ ligands (Lord

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et al., 1977), can indirectly modulate μ-mediated effects through actions at the δ receptor. Intracerebroventricular (i.c.v.) administration of sub-antinociceptive doses of [Leu^5]enkephalin, as well as [Leu^5]enkephalin analogs, have been shown to increase i.c.v. morphine antinociceptive potency in the mouse (Vaught and Takemori, 1979; Barrett and Vaught, 1982; Vaught et al., 1982). Conversely, sub-antinociceptive doses of [Met^5]enkephalin and [Met^5]enkephalin analogs significantly decrease i.c.v. morphine antinociceptive potency (Lee et al., 1980; Vaught et al., 1982). Evidence that these modulatory effects are mediated via a δ receptor includes the recent observations that the δ-selective antagonist ICI 174,864 (Cotton et al., 1984) prevented both the increase (Heyman et al., 1986b; 1989) and the decrease (Heyman et al., 1986b) of morphine antinociception produced by sub-agonist doses of the δ-selective [D-Pen^2,D-Pen^5]enkephalin (DPDPE) (Mosberg et al., 1983; Porreca et al., 1984; James and Goldstein, 1984) and [D-Ala^2,Met^5]enkephalinamide (DAMA), respectively; ICI 174,864 had no direct effect on morphine antinociception (Heyman et al., 1987).

The ability of δ agonists to produce antinociception directly, as well as to modulate (i.e. increase or decrease) μ-mediated antinociceptive potency, may imply the existence of subclasses of δ and μ receptors. Early ligand binding studies demonstrating apparent non-competitive interactions between μ and δ binding sites (Rothman and Westfall, 1982a,b) led to the hypothesis that the modulatory effects of δ agonists on μ-mediated antinociception occur through a δ binding site of an opioid receptor complex made up of distinct, yet interacting μ and δ binding sites (Vaught et al., 1982). More recent ligand binding studies have refined this hypothesis and have shown that the δ agonist, [^{3}H][D-Ala^2,D-Leu^5]enkephalin ([^{3}H]DADLE) labels two binding sites in vitro which are distinguished by the inhibitory mechanism of μ ligands (Rothman et al., 1985a,c); while μ ligands are weak, competitive inhibitors at the higher affinity [^{3}H]DADLE binding site, commonly identified as δ, they are potent, non-competitive inhibitors at the lower affinity [^{3}H]DADLE binding site. Based on the potent displacement of [^{3}H]DADLE from the lower affinity site, Chang and Cuatrecasas (1979) identified it as a μ binding site. However, a number of experimental manipulations demonstrate differences between μ binding sites and the lower affinity [^{3}H]DADLE binding site. These include: (a) the ionic composition of the assay medium (Bowen et al., 1981; Rothman et al., 1984b); (b) the ability of the site directed acylating agent FIT (N-phenyl-N-[1-(2-(p-isothiocyanato)phenylethyl-4-piperadinyl]propanamide)-HCl to unmask lower affinity [^{3}H]DADLE binding sites but not μ sites (Rothman et al., 1985b); (c) the observation that the i.c.v. administration of β-funaltrexamine (β-FNA, Portoghese et al., 1980) to rats 24 h prior to preparation of brain membranes results in an approximately 60% decrease in the B_{max} of the lower affinity [^{3}H]DADLE binding site without any alteration in [^{3}H][D-Ala^2,NMPhe^4,Gly-ol]enkephalin ([^{3}H]DAGO) (Handa et al., 1981) binding parameters (Rothman et al., 1984a; 1986; 1989) and (d) the finding that μ ligands are non-competitive inhibitors of a μ binding site labelled by [^{3}H]naloxone (Rothman et al., 1985c) and [^{3}H]17-cyclopropylmethyl-3,14-dihydroxy-4,5-α-epoxy-6-β-fluoro-morphinan-([^{3}H]cycloFOXY) (Rothman et al., 1987b).

These reciprocal non-competitive interactions between μ and δ receptors led to the suggestion that (a) the lower affinity [^{3}H]DADLE binding site is the δ binding site of an opioid receptor complex (termed δ_{cx} to indicate 'in the complex') and that the non-competitive interaction of μ ligands on the lower affinity [^{3}H]DADLE binding site is mediated through an adjacent μ binding site (termed μ_{cx} to indicate in the complex); (b) the higher affinity [^{3}H]DADLE binding site is a δ binding site which is not part of the receptor complex (termed δ_{ncx} to indicate 'not in the complex'), and (c) that μ-mediated antinociception occurs via binding of μ ligands to the μ_{cx} binding site while the modulatory effects of δ agonists on μ-mediated antinociception is mediated via binding to the δ binding site of the opioid receptor complex (δ_{cx}).

The present investigation was designed to further elucidate the possible functional interaction of μ and δ receptors in the production of antinociception and to test the hypothesis that the

modulatory effects of δ agonists on morphine antinociception are mediated via actions at the δ_{ex} receptor. Recent studies in the rat have suggested that the irreversible μ antagonist, β-FNA may provide a means to test this hypothesis, as this compound has been shown to alkylate the receptor complex (Rothman et al., 1986; 1989). Thus, the μ modulatory effects of DPDPE and DAMA, δ agonists previously shown to potentiate and antagonize morphine antinociception, respectively, were studied in the presence of the selective opioid antagonist, β-FNA (Portoghese et al., 1980). Additionally, in an attempt to gain further insight into the nature of the μ_{ex} and μ_{nex} sites, naloxonazine (Hahn et al., 1982), an antagonist of the putative μ_1 receptor (Pasternak et al., 1980) was studied.

2. Materials and methods

2.1. Animals

Male, ICR mice (20-30 g, Harlan, Indianapolis, IN) were used for all experiments. Animals were kept in groups of five in a temperature-controlled room with a standard 12 h light/dark cycle (lights on 07:00 h). Food and water were continuously available.

2.2. Injection techniques

Compounds were delivered into the lateral cerebral ventricle using a modification of the method of Haley and McCormick (1957) as previously described (Porreca et al., 1984). Briefly, the mice were lightly anesthetized with ether, an incision was made in the scalp and bregma located. The injections were made 2 mm caudal and 2 mm lateral to bregma at a depth of 3 mm using a Hamilton (Reno, NV) microliter syringe with a 26 gauge needle. All i.c.v. injections were made in a volume of 5 μl.

2.3. Antagonist pretreatment

In studies employing β-FNA, each mouse received a single i.c.v. injection of distilled water (vehicle) or β-FNA (18 nmol) 4 h prior to agonist administration, similar to the procedure described by Ward et al. (1982). In studies with naloxonazine, each mouse received a single subcutaneous (s.c.) injection of distilled water (vehicle) or naloxonazine HCl (35 mg/kg) 24 h prior to testing as described by Ling et al. (1986).

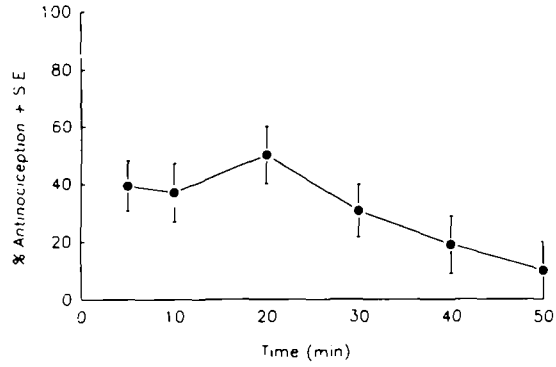

Fig. 1. Time-response curve for i.c.v. DAMA (1.7 nmol) antinociception in the mouse. Data are means and S.E.

2.4. Antinociceptive testing

Antinociceptive responses were determined using warm (55°C) water as the nociceptive stimulus where the latency to tail withdrawal was taken as the endpoint (Janssen et al., 1963). Prior to agonist administration, the tail of each mouse was immersed in the water and the latency to a rapid flick recorded. Animals not flicking their tails within 5 s were eliminated from the study. This procedure was repeated 20 min after i.c.v. administration of morphine, DPDPE and DAMA; this was the time of peak agonist effect as determined from time-response curves (morphine and DPDPE, Heyman et al., 1986a; DAMA, fig. 1). Animals not flicking their tails within 15 s were removed from the nociceptive stimulus and assigned a maximal score of 100% in order to avoid tissue damage. Antinociception was expressed as: % antinociception = 100 × (test latency − control latency)/(15 s − control latency). All testing was done in unanesthetized mice.

Following the determination of the i.c.v. dose-response curves for DPDPE, morphine and DAMA in control (DPDPE and morphine, Hey-

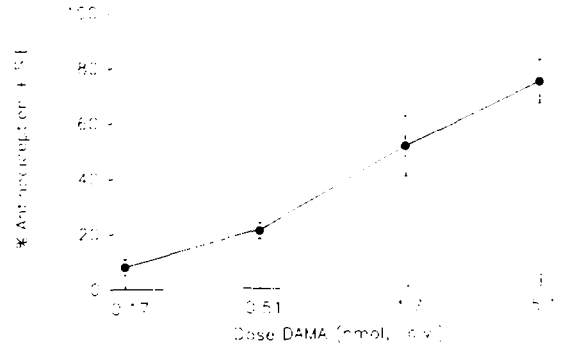

Fig. 2. Dose-response relationship of i.c.v. DAMA antinociception in the mouse when tested at 20 min post-injection. Data are means and S.E.

man et al., 1986a; DAMA, fig. 2), β-FNA (DPDPE and morphine, Heyman et al., 1987; DAMA, fig. 3) and naloxonazine (DPDPE and morphine, Heyman et al., 1988; DAMA, fig. 3), pretreated mice, doses of DPDPE and DAMA which produced barely detectable antinociception (0-5%) in the respective groups were chosen by downward extrapolation of the dose-response line. In order to determine if DPDPE and/or DAMA were capable of modulating i.c.v. morphine antinociception in animals pretreated with β-FNA or naloxonazine, the δ agonists were co-administered in the same i.c.v. injection with morphine as previously described by Vaught et al. (1982). Testing took place 20 min after injection.

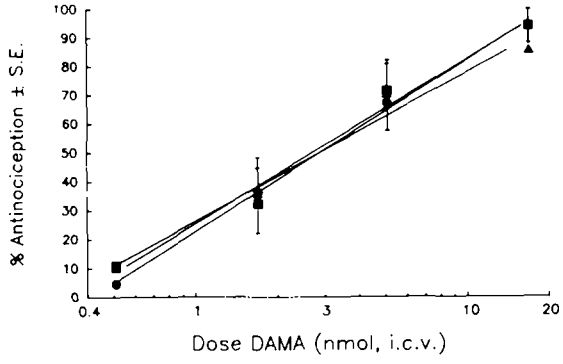

Fig. 3. Effects of pretreatment with β-FNA (18 nmol i.c.v. at −4 h) (▲) or naloxonazine (35 mg/kg s.c. at −24 h) (■) on i.c.v. DAMA (●) antinociception. Data are means and S.E.

2.5. Chemicals

DPDPE and DAMA (Peninsula Laboratories, Inc., San Carlos, CA) were dissolved in distilled water, frozen in aliquots and lyophilized, and redissolved immediately before use. Morphine sulfate (Mallinckrodt Inc., St. Louis, MO), β-FNA (Research Biochemicals Inc., Wayland, MA) and naloxonazine HCl were dissolved in distilled water just prior to administration. Naloxonazine was a generous gift of Dr. Diane DeHaven (Nova Pharmaceutical Co., Baltimore, MD).

2.6. Statistics

The doses of DPDPE and DAMA chosen for the modulation of morphine antinociception were extrapolated from regression lines determined by plotting each individual point using the computer program of Tallarida and Murray (1986) (procedure 8) in control, β-FNA and naloxonazine pretreated mice. A minimum of 10 mice were studied at each dose level. Modulatory effects of DPDPE and DAMA on morphine antinociception were identified using a Student's t-test for grouped data. The data are presented as the mean and the error bars are the S.E.

3. Results

3.1. Studies in mice pretreated with β-FNA

The first study examined the effects of β-FNA pretreatment (18 nmol i.c.v., at −4 h) on the respective ability of DPDPE to increase, and DAMA to decrease, i.c.v. morphine antinociceptive potency. As the β-FNA pretreatment used in the current study had no significant effect on the direct antinociceptive effects of i.c.v. DPDPE (Heyman et al., 1987; present study) or DAMA (fig. 3), the modulatory doses for each δ agonist (1.6 nmol DPDPE and 0.17 nmol DAMA) remained the same. As expected (Ward et al., 1982; Ward and Takemori, 1983; Heyman et al., 1987), however, morphine antinociception was significantly antagonized by β-FNA pretreatment; thus, the doses of morphine used in β-FNA pretreated

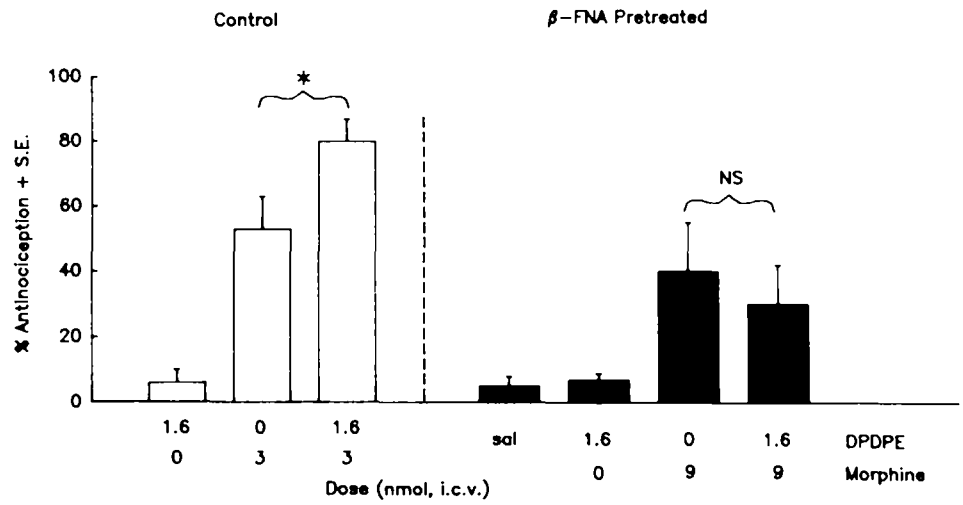

Fig. 4. Effects of β-FNA pretreatment (18 nmol at −4 h) on DPDPE-induced modulation of morphine antinociception. β-FNA pretreatment abolishes the increase of morphine antinociceptive potency produced by DPDPE. Data shown are means and S.E. and asterisks indicate a significant difference from control ($P < 0.05$, Student's t-test).

mice were increased by 10-fold (3 and 30 nmol, respectively, in naive and β-FNA groups shown in figs. 4 and 5). Pretreatment with the μ antagonist β-FNA blocked the modulatory effect of DPDPE (fig. 4) and that of DAMA (fig. 5) when compared to control (5 μl distilled water i.c.v., at −4 h) groups. β-FNA had no antinociceptive effect alone in this test.

3.2. Studies in mice pretreated with naloxonazine

Naloxonazine pretreatment (35 mg/kg s.c. at −24 h) had no effect on the direct antinociceptive effect produced by i.c.v. DPDPE (fig. 6), in agreement with previous results (Heyman et al., 1988), or DAMA (fig. 3); thus, no changes were required in the modulatory doses of these δ agonists. As

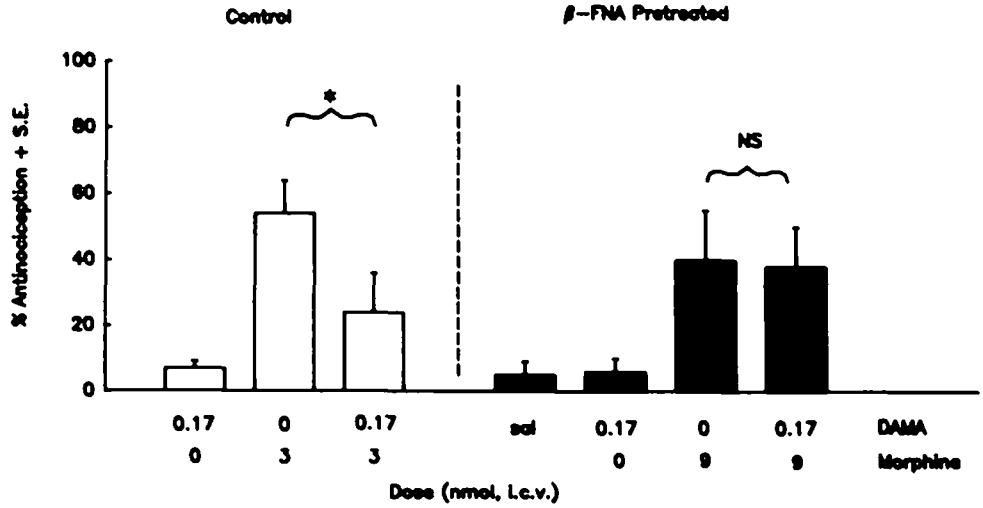

Fig. 5. Effects of β-FNA pretreatment (18 nmol at −4 h) on DAMA-induced modulation of morphine antinociception. β-FNA pretreatment abolishes the attenuation of morphine antinociception by DAMA. Data shown are means and S.E. and asterisks indicate a significant difference from control ($P < 0.05$, Student's t-test).

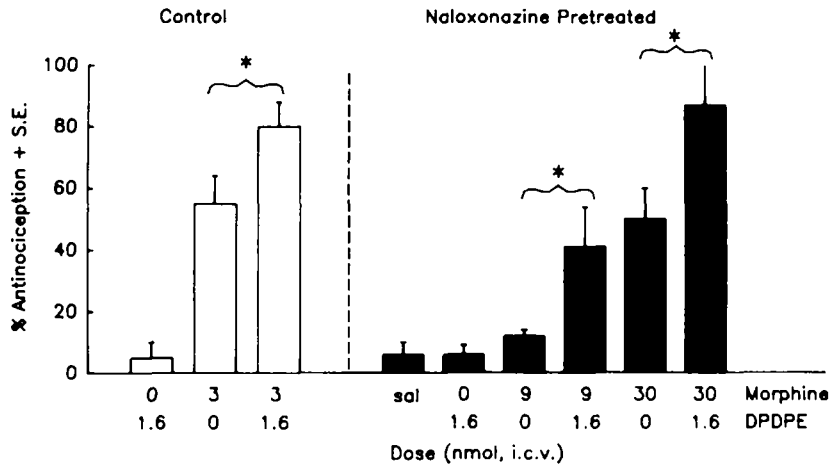

Fig. 6. Effects of naloxonazine pretreatment (35 mg/kg s.c. at −24 h) on DPDPE-induced modulation of morphine antinociception. Naloxonazine pretreatment does not affect the increase in potency of morphine resulting from DPDPE. Data are means and S.E. and asterisks indicate a significant difference from controls ($P < 0.05$, Student's t-test).

expected (Ling et al., 1986; Heyman et al., 1988), pretreatment with naloxonazine effectively antagonized i.c.v. morphine antinociception (figs. 6 and 7) and so the doses of morphine used were increased by 10-fold. Unlike the effects of pretreatment with β-FNA, naloxonazine pretreatment did not alter the ability of DPDPE (fig. 6) or of DAMA to modulate (fig. 7) morphine antinociception.

4. Discussion

The present study attempted to further our understanding of μ-δ interactions in the produc-

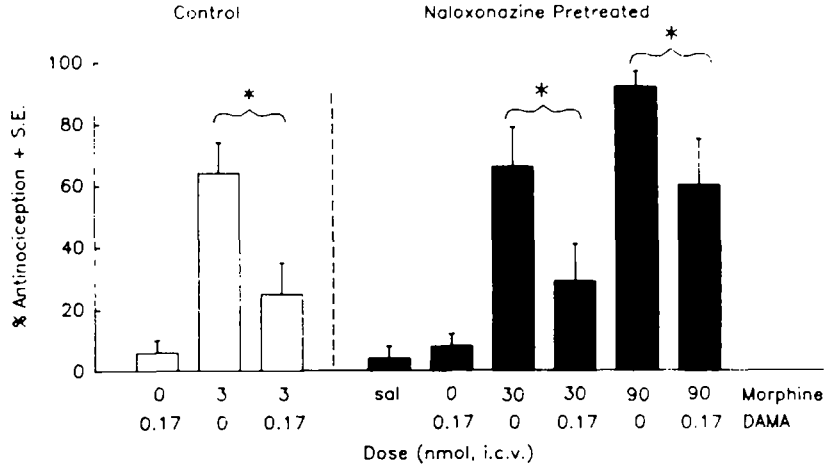

Fig. 7. Effects of naloxonazine pretreatment (35 mg/kg s.c. at −24 h) on DAMA-induced modulation of morphine antinociception. Naloxonazine pretreatment does not affect the attenuation of morphine antinociception produced by DAMA. Data are means and S.E. and asterisks indicate a significant difference from controls ($P < 0.005$, Student's t-test).

tion of antinociception using receptor selective antagonists. Doses of the δ agonists DPDPE and DAMA which produced no significant antinociception when given alone were found to increase and decrease the potency of i.c.v. morphine in producing antinociception, respectively, in agreement with previous studies (Lee et al., 1980; Vaught et al., 1982; Porreca et al., 1987; Heyman et al., 1986b; 1989). These modulatory effects have previously been shown to be due to an interaction of the agonists with the δ receptors as the δ-selective antagonist ICI 174,864 (Cotton et al., 1984) prevented both the increase (Heyman et al., 1986b; 1988b) and decrease (Heyman et al., 1986b) of morphine potency produced by DPDPE and DAMA, respectively; ICI 174,864 did not directly antagonize morphine antinociception (Heyman et al., 1987).

Reasoning, therefore, that morphine antinociception may be modulated in part via a hypothesized μ-δ complex, attempts to disrupt this interaction were made using the non-equilibrium μ-selective antagonist, β-FNA (Portoghese et al., 1980; Ward and Takemori, 1983). Previous work has shown that β-FNA pretreatment prevents the ability of ICI 154,129, a δ-selective antagonist (Shaw et al., 1982) to reverse endotoxic shock (Holaday and D'Amato, 1983; D'Amato and Holaday, 1984). Reasonable μ selectivity has been demonstrated for β-FNA, however, as this antagonist does not inhibit δ_{ncx} binding in rat brain membrane preparations (Rothman et al., 1984a; 1987a,b), nor does it block the antinociceptive effects of δ agonists given i.c.v. in mice (Heyman et al., 1987). Thus, the ability of β-FNA to prevent the effects of ICI 154,129 was suggested to be the result of an alteration in the μ-δ receptor complex (Holaday and D'Amato, 1983; D'Amato and Holaday, 1984). Additionally, i.c.v. administration of β-FNA to rats 18-24 h prior to preparation of brain membranes has been demonstrated to lead to a selective alkylation of the opioid receptor complex which was reflected by a substantial decrease in the B_{max} of the δ_{cx} binding site, while the binding of [^{3}H]DADLE to the δ_{ncx} site was not significantly altered (Rothman et al., 1986; 1989). This apparent selective effect of β-FNA on the opioid receptor complex in vivo allowed the formulation of the following testable prediction: if the modulatory effects of sub-antinociceptive doses of δ agonists on morphine antinociception are mediated via an opioid receptor complex, then pretreatment with β-FNA should prevent the ability of δ agonists to modulate morphine antinociception.

The present study demonstrates that pretreatment with β-FNA abolishes both the increase and decrease of morphine potency associated with DPDPE and DAMA, respectively, a finding which might be interpreted as supporting the concept of functional uncoupling of the μ-δ complex given the observation of disruption of the opioid receptor complex by β-FNA in vivo (Rothman et al., 1986; 1989). An alternative explanation seems possible, however, as previous studies have shown that after blockade of available μ receptors with β-FNA, morphine produces its antinociception at a non-μ site (Heyman et al., 1987; Takemori and Portoghese, 1987). Takemori and Portoghese (1987) have shown that the naloxone apparent pA_2 value against morphine in the mouse abdominal stretch test changes significantly after β-FNA treatment suggesting that morphine interacts with non-μ (δ and κ) receptors to produce antinociception. Additionally, Heyman et al. (1987) and Takemori and Portoghese (1987) have shown that the selective δ antagonists ICI 174,864 (Cotton et al., 1984) and ICI 154,129 (Shaw et al., 1982), respectively, compounds which do not directly antagonize morphine tail withdrawal antinociception in control mice, significantly antagonize morphine antinociception in β-FNA-pretreated mice. Modulation by δ agonists, therefore, would not be possible if both agonists were acting at the same receptor (δ_{ncx}). Furthermore, the competitive interactions of μ and δ ligands at this binding site (δ_{ncx}) in vitro are consistent with the observations in vivo which demonstrate that DPDPE and DAMA do not modulate morphine antinociception when morphine acts at the δ_{ncx} site.

Further attempts to characterize the μ-δ functional interaction were made using the long-lasting proposed μ_1 antagonist naloxonazine. Naloxonazine pretreatment had no effect on i.c.v. DPDPE antinociception in agreement with previous studies where this antagonist neither blocked i.c.v. DPDPE

antinociception in the mouse (Heyman et al., 1988) or rat (Ling et al., 1986), nor altered binding of DPDPE in rat brain preparations (Clark et al., 1986). Similarly, naloxonazine pretreatment had no effect on i.c.v. DAMA antinociception (present study). In contrast to the lack of effect of naloxonazine on DPDPE and DAMA antinociception, this antagonist exhibited the expected long-lasting blockade of i.c.v. morphine antinociception in the mouse in agreement with previous studies in this species (Heyman et al., 1988) as well as in the rat (Ling et al., 1986). Potentiation and attenuation of morphine antinociception by DPDPE and DAMA, respectively, were still evident in mice that received naloxonazine treatment prior to testing. Various interpretations might be made from these findings. For example, naloxonazine might alter the conformation of the receptor complex in such a way that morphine binds with lower affinity, thus reducing the potency, but leaving the modulatory mechanisms intact. As the mechanism of action for the long-lasting antagonism associated with naloxonazine is not well understood, continued action of morphine may be due to incomplete blockade of μ_{cx} receptors. Alternatively, it is also possible that the μ receptors in the receptor complex are naloxonazine insensitivity (i.e., μ_2).

As is often the case, the present findings in vivo are not in complete agreement with hypotheses drawn from findings in vitro. Based on evidence from binding studies with β-FNA, one would predict that all μ agonists act at the μ_{cx} receptor to produce their antinociception (Rothman et al., 1986; 1987a; 1989). It has been shown previously (Heyman et al., 1989), however, that while morphine antinociception is potentiated by DPDPE, the antinociceptive effects of the μ agonist DAGO (Handa et al., 1981) are not, a finding which suggests that morphine and DAGO may act at two distinct μ receptors (μ_{cx} and μ_{ncx} respectively) to produce their antinociceptive effects. Although morphine and DAGO antinociception are both antagonized by pretreatment with β-FNA (Heyman et al., 1987), a finding in agreement with binding studies and the above prediction, only morphine antinociception is modulated by DPDPE (Heyman et al., 1989). The lack of modulation of DAGO antinociception by DPDPE, therefore, is not directly in accord with the concept that the μ_{cx} receptor is the sole site for the mediation of μ antinociception. It is also important to note that while naloxonazine pretreatment has previously been shown to antagonize both i.c.v. morphine and DAGO antinociception (Heyman et al., 1988), this antagonist had no effect on the ability of DPDPE or DAMA to modulate morphine antinociception. The lack of effect of naloxonazine on the modulation of morphine antinociception coupled with the antagonism of both morphine and DAGO antinociception by naloxonazine again suggests a discrepancy with the concept that antinociception is mediated solely at the μ_{cx} site. Although reasons which might account for these discrepancies can be formulated, it seems clear that further experiments are needed to resolve the inconsistencies noted above. Although inconsistencies do exist as to the identity of the specific site where μ agonists act to produce their antinociceptive effects, it is important to note that the differential antagonism of the modulation observed with β-FNA and naloxonazine, nevertheless continues to support the concept of μ receptor subtypes.

Although the existence of such a complex remains tentative, μ-δ interactions have been demonstrated for other endpoints from studies in vivo and in vitro. In addition to antinociception, μ-δ interactions have also been observed in vivo in the reversal of endotoxic shock (Holaday and D'Amato, 1983; D'Amato and Holaday, 1984; Holaday et al., 1985; 1986) and the elevation of flurothyl seizure threshold (Holaday et al., 1985). The finding that β-FNA, but not naloxonazine, affected the δ modulation of μ antinociception provides support in vivo for the concept that morphine antinociception is mediated through this complex, that δ modulation of morphine antinociception occurs through this complex and that the μ site in the receptor complex is naloxonazine insensitive. Thus, in addition to playing a direct role in the production of antinociception, the enkephalins may also play a modulatory role in antinociception by acting in an opioid (μ-δ) receptor complex.

Acknowledgements

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