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Interaction between the μ-agonist dermorphin and the δ-agonist [D-Ala^2, Glu^4]deltorphin in supraspinal antinociception and δ-opioid receptor binding

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1 In rats, the interaction between the μ-opioid agonist dermorphin and the δ-opioid agonist [D-Ala^2, Glu^4]deltorphin was studied in binding experiments to δ-opioid receptors and in the antinociceptive test to radiant heat.

2 When injected i.c.v., doses of [D-Ala^2, Glu^4]deltorphin higher than 20 nmol produced antinociception in the rat tail-flick test to radiant heat. Lower doses were inactive. None of the doses tested elicited the maximum achievable response. This partial antinociception was accomplished with an *in vivo* occupancy of more than 97% of brain δ-opioid receptors and of 17% of μ-opioid receptors. Naloxone (0.1 mg kg^{-1}, s.c.), and naloxonazine (10 mg kg^{-1}, i.v., 24 h before), but not the selective δ-opioid antagonist naltrindole, antagonized the antinociception.

3 *In vitro* competitive inhibition studies in rat brain membranes showed that [D-Ala^2, Glu^4]deltorphin displaced [^{3}H]-naltrindole from two δ-binding sites of high and low affinity. The addition of 100 μM Gpp[NH]p produced a three fold increase in the [D-Ala^2, Glu^4]deltorphin K_i value for both binding sites. The addition of 10 nM dermorphin increased the K_i value of the δ-agonist for the high affinity site five times. When Gpp[NH]p was added to the incubation medium together with 10 nM dermorphin, the high affinity K_i of the δ-agonist increased 15 times.

4 Co-administration into the rat brain ventricles of subanalgesic doses of dermorphin and [D-Ala^2, Glu^4]deltorphin resulted in synergistic antinociceptive responses.

5 Pretreatment with naloxone or with the non-equilibrium μ-antagonists naloxonazine and β-funaltrexamine completely abolished the antinociceptive response of the μ-δ agonist combinations.

6 Pretreatment with the δ-opioid antagonists naltrindole and DALCE reduced the antinociceptive response of the dermorphin-[D-Ala^2, Glu^4]deltorphin combinations to a value near that observed after the μ-agonist alone. At the dosage used, naltrindole occupied more than 98% of brain δ-opioid receptors without affecting μ-opioid-receptors.

7 These data suggest that in the rat tail-flick test to radiant heat, μ- and δ-opioid agonists co-operate positively in evoking an antinociceptive response. Although interactions between different opioid pathways cannot be excluded, *in vitro* binding results indicate that this co-operative antinociception is probably mediated by co-activation of the δ-opioid receptors at the cellular level by the μ- and δ-agonist.

Keywords: Dermorphin; [D-Ala^2, Glu^4]deltorphin; synergistic interaction; δ-opioid binding; antinociception

Introduction

Evidence has accumulated to support the hypothesis that μ- and δ-opioid agonists interact in a co-operative manner. For instance, μ-δ cooperation has been reported in the opioid inhibition of gut propulsion (Heyman, 1987) and of urinary bladder contractions (Sheldon *et al.*, 1989) and in the opioid-induced changes of EEG and EEG spectral power (Stamidis & Young, 1992). Holaday and D'Amato (1983) described μ-δ interactions in the modulation of endotoxic shock in the rat and Kamei *et al.* (1993) demonstrated modulation of the antitussive activity of μ-opioid receptor agonists by δ-opioid agonists in mice. In rats, pretreatment with morphine or with the selective μ-agonist dermorphin produced sensitization to the behavioural effects of the selective δ agonist [D-Ala^2, Glu^4]deltorphin (Melchiorri *et al.*, 1992). Agonists at the δ-opioid receptor can also modulate the antinociception of μ-agonists such as morphine and normorphine (Vaught & Takemori, 1979; Porreca *et al.*, 1987; Heyman *et al.*, 1989; Jiang *et al.*, 1990). In a more rigorous isobolographic analysis of the μ-δ interaction in the mouse, Porreca *et al.* (1992) and Horan *et al.* (1992) tested fixed ratio combinations of morphine with the δ agonists [D-Pen^2, D-Pen^5]enkephalin (DPDPE) and [D-Ala^2, Glu^4]deltorphin in the hot water tail-flick test and found significant superadditive antinociception. When the antinociceptive response was measured in the rat with the cold water tail-flick test, Adams *et al.* (1993) found a positive co-operation between DPDPE and morphine. Surprisingly, a μ-δ interaction was not observed with μ agonists other than morphine and normorphine. In mice antinociceptive responses to sufentanil, meperidine, methadone and to the μ-selective peptides [D-Ala^2, MePhe^4, Gly-ol^5]enkephalin (DAMGO) and PLO17 were not affected by DPDPE (Heyman *et al.*, 1989). Also in rats, isobolographic analysis failed to reveal co-operative interactions between DPDPE and PLO17 in the cold water and hot water tail-flick tests, though both the peptides did act as full agonists in the cold water test (Adams *et al.*, 1993). Another problem arises from the fact that antinociceptive tests are not all equally sensitive to δ-opioid agonists, whereas they are fully sensitive to μ-agonists. For example, the hot-plate test in mice and rats, the cold water tail-flick test in rats and hot water tail-flick test in mice are sensitive to δ-opioid agonists, whereas the tail-flick tests to hot water or radiant heat in rats are scarcely or not sensitive (Heyman *et al.*, 1987; Calcagnetti *et al.*, 1990b; Negri *et al.*, 1991). Thus, when pain was produced in rats by immersing the tail in hot water no

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synergism between morphine and DPDPE was evident (Adams *et al.*, 1993). Likewise, in the rat tail-flick test to radiant heat (D'Amour & Smith, 1941) positive co-operative antinociception between μ-selective and δ-selective opioid peptides has not been observed.

Nevertheless, there is evidence that even in tests that are usually not sensitive to δ-opioid agonists alone, co-activation of the endogenous opioid system by animal handling, environmental factors, stress or chronic pain permits δ-opioid agonists to produce some degree of antinociception (Calcagnetti *et al.*, 1990b). Though the type of opioid receptors involved in the modulation of this apparently δ-mediated antinociception remains unestablished, the results obtained with opioid antagonists or with μ-receptor deficient animals indicate that the endogenous μ-opioid system plays a primary role in modulating the antinociceptive responses to exogenous δ-agonists (Raffa *et al.*, 1992).

In conclusion, the opioid-receptor type mainly involved in the supraspinal antinociception produced by combinations of μ- and δ-agonists remains a matter of debate.

Evidence of interaction between μ- and δ-agonists comes also from receptor binding studies. Rothman and Westfall (1982a,b; Rothman *et al.*, 1988) demonstrated an apparent non competitive interaction between μ and δ binding sites and suggested that δ-receptors can exist either separately or in a physically associated state with μ-receptors. These separate or associated δ-receptors were termed the δ_{ncx} (δ non-complexed receptor) and δ_{cx} (δ complexed receptor). More recent ligand binding studies showed that [D-Ala^2, Glu^4]deltorphin selectively binds the δ_{cx} receptor (Cha *et al.*, 1994).

In the present investigation we used as selective agonists for μ- and δ-opioid receptors the peptides dermorphin and [D-Ala^2, Glu^4]deltorphin, respectively. These two heptapeptides share the N-terminal sequence Tyr-D-Ala-Phe which is considered to represent the addressing non-selective domain for opioid receptor binding (Erspamer *et al.*, 1989). This common domain may provide the structural requirements for receptor interactions. Thus the investigation was designed to demonstrate that the interaction between these two opioid agonists takes place both *in vitro*, at the receptor level and *in vivo*, in the rat supraspinal antinociception. In binding experiments we sought evidence for a co-operative activation of brain δ-opioid receptors by combinations of dermorphin with [D-Ala^2, Glu^4]deltorphin. To test antinociception, using the rat tail-flick test to radiant heat, we co-administered [D-Ala^2, Glu^4]deltorphin with subanalgesic doses of the μ-agonist dermorphin. To elucidate further the types of supraspinal opioid receptors involved in this antinociceptive co-operation, we used selective antagonists at μ- or δ-sites in combination with dermorphin-[D-Ala^2, Glu^4]deltorphin mixtures.

Methods

In vivo *opioid receptor occupancy*

Before being killed for binding studies, groups of five rats each were injected i.c.v. with saline, naltrindole (2 nmol, 90 and 5 min before), or [D-Ala^2, Glu^4]deltorphin (13 or 60 nmol, 20 min before). Rats were killed by guillotine. The whole brain minus cerebellum was removed, weighed and homogenized in 100 volumes of ice-cold 50 mM Tris-HCl buffer (pH 7.4). Homogenates were then centrifuged (4°C, 33000 *g*) and the pellets resuspended and centrifuged again. The entire procedure from brain removal to final pellets lasted not more than 30–35 min. Pellets were then stored at −70°C and resuspended in 50 volumes of ice-cold 50 mM Tris-HCl buffer (pH 7.4), immediately before binding assay. Each assay contained, in a final volume of 2 ml, the membrane preparation (0.8–1.0 mg of proteins, equivalent to 20 mg of brain wet tissue) and the tritiated ligand. The μ-binding site was selectively labelled with [^{3}H]-DAMGO ([^{3}H]-[D-Ala^2, MePhe^4, Gly-ol^5]enkephalin); the δ-binding site with [^{3}H]-[D-Ala^2, Glu^4]deltorphin. After a 90 min incubation at 35°C the samples were cooled at 4°C and the free ligand was separated, from membrane-bound ligand, by filtration under reduced pressure over Whatman GF/B filters (soaked in 0.1% bovine serum albumin incubation buffer, for 1 h), followed by three washings with 5 ml of ice-cold buffer. Radioactivity was extracted in 10 ml of Filter-Count scintillation cocktail (Packard Instrument Company, Inc., Downers Grove, Il) and measured in a liquid scintillation counter (Betamatic, Kontron). Saturation curves of tritiated peptide ligands were performed in triplicate. Binding parameters (K_i, and B_{max}) were estimated by use of nonlinear regression (LIGAND, Biosoft, Cambridge, U.K.). To ascertain whether some amount of drug was washed away from receptor sites during membrane preparation, we prepared membranes from brain homogenates which had been previously incubated with [^{3}H]-[D-Ala^2, Glu^4]deltorphin. In brief, a whole brain minus cerebellum was removed from a saline-injected rat and homogenized in 100 volumes of ice-cold 50 mM Tris-HCl buffer (pH 7.4) and membranes were prepared as previously described. Half the membranes were incubated for 90 min in 50 mM Tris-HCl buffer (pH 7.4) containing 3 nM [^{3}H]-[D-Ala^2, Glu^4]deltorphin. To measure non-specific binding, the remaining membranes were incubated in the Tris-HCl buffer to which 3 nM [^{3}H]-[D-Ala^2, Glu^4]deltorphin and 50 μM naloxone were added. After incubation, the samples were cooled and both membrane sets were divided into two equal aliquots. One aliquot was centrifuged (4°C, 33000 *g*) and the pellets were resuspended and centrifuged again. The final pellets were resuspended in Tris buffer and filtered over GF/B filters (washed membrane). The other aliquot was immediately filtered without dilution and washing, (unwashed membranes). Filters were then washed three times with ice-cold buffer. The specific binding of the washed membranes was calculated and compared with that of unwashed membranes. Another set of binding experiments was performed to verify whether during brain homogenization an amount of the injected drug, still diffusing to receptor sites or free in ventricle, was artificially exposed to sites that it would not reach *in vivo* after administration for behavioral testing. The whole brain minus cerebellum of saline-injected rats was homogenized in 100 volumes of ice-cold 50 mM Tris-HCl buffer (pH 7.4) to which 10 nmol of [^{3}H]-[D-Ala^2, Glu^4]deltorphin were added. Membrane pellets were immediately prepared as described. Samples of pellets (0.8–1.0 mg of proteins) were resuspended in 2 ml Tris-HCl buffer, filtered and counted in the liquid scintillation spectrometer. The remaining pellets were resuspended in Tris-HCl buffer (0.8–1.0 mg of proteins per 2 ml), incubated for 90 min at 35°C with 50 mM naloxone, filtered and counted to measure non-specific binding.

Inhibition of [^{3}H]-naltrindole binding

Brain membranes to be used for [^{3}H]-naltrindole inhibition experiments were preincubated at 25°C for 30 min to remove endogenous ligands. δ-Opioid receptors were labelled with 0.1 nM [^{3}H]-naltrindole in Tris-HCl pH 7.4, containing 100 mM NaCl (35°C, 90 min). Each displacement curve of [^{3}H]-naltrindole binding was obtained with 14 graded concentrations of [D-Ala^2, Glu^4]deltorphin or naltrindole, made in triplicate. In experiments designed to study the effects of guanosine 5'-triphosphate (GTP) or dermorphin on binding parameters, Gpp[NH]p (100 μM) or dermorphin (10 nM) were added to the incubation medium. Binding parameters (K_i and B_{max}) were estimated by use of nonlinear regression (LIGAND, Biosoft, Cambridge, U.K.). To calculate binding parameters pooled membranes from five brains were used to draw the seven displacement curves. This set of curves was repeated five times with five different pools of membranes. Thus the K_i value (mean ± s.e.mean) of each displacement paradigm was calculated from five curves. K_i values from different displacement experiments were then compared with a two-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test.

Animals and surgery

Male Wistar rats weighing 240–260 g were used. Under light ethyl ether anaesthesia each rat was implanted surgically with a plastic guide cannula, 7 mm in length, (Linca, Tel-Aviv, Israel), stereotaxically inserted through a skull-hole drilled over the left lateral ventricle of the brain, as previously described in detail (Negri *et al.*, 1995). After surgery, the rats were allowed to recover for 4 to 7 days in individual plastic cages. Food and water were available *ad libitum* and the animals were maintained on a natural day/night, light/dark cycle. A 10 μl Hamilton syringe fitted with a 26 gauge needle was used for i.c.v. injections. The needle was inserted through the guide cannula to a depth of 3.6 mm below the external surface of the skull in awake rats. Drugs and control solutions were injected slowly (60 s) in a constant volume of 5 μl. To avoid stress-induced analgesia, rats were accustomed to being handled for three days before experiments. The IASP guidelines on ethical standards for investigations for experimental pain in animals were followed. At the end of the experimental session, rats were killed by inspiration of CO_2 at a concentration of 75% in air.

Test of antinociception

Analgesia was measured by the tail-flick test to radiant heat (D'Amour & Smith, 1941). The latency to tail withdrawal was taken as a measure of the nociceptive response to heat exposure. The intensity of the thermal stimulus (a light beam from a 100 watt, 20 V bulb, focused on the tail tip) was adjusted to obtain a predrug latency ranging from 2 to 4 s. Three predrug latencies were measured at 30 and 15 min and immediately before drug injection. The first reading was discarded and the second two were averaged to determine the base-line latency (CL). Animals not flicking their tails within 4 s were discarded. The test was repeated at 15 min intervals during the first hour after drug administration and every 30 min thereafter for a total period of 4 h. The latency to tail-flick of each drug-injected animal was defined as the test latency (TL). To avoid tissue damage, animals with a test latency of more than 12 s (cut-off time), were removed from the nociceptive stimulus and assigned a TL value of 12. For drawing the dose- and time-response curves, the antinociceptive response was expressed as MPE, calculated by the following equation:

$$\text{MPE} = 100 \times (\text{TL} - \text{CL})/(12 - \text{CL})$$

A computer program (PRISM, GraphPad, CA, U.S.A.) was used to calculate the area under the time-response curve (AUC) for each animal and for all opioid doses. For each dose, the antinociceptive response was expressed as the mean MPE ± s.e.mean or mean AUC ± s.e.mean. The maximum effect was defined as the AUC value (AUC_{max}) when the peak effect was equal to 100 MPE. A_{50} was defined as the opioid dose that produced an AUC equal to 50% of the maximum effect.

Experimental design

To determine the antinociceptive dose-response curve for each single agonist in the tail-flick test the following doses were injected i.c.v.: 1.0, 3.7, 7.4, 9.9, 13, 18, 30, 60 and 90 nmol of [D-Ala^2, Glu^4]deltorphin; 0.1, 0.3, 0.7, 1.2, 2.5, 6.2, 12.4, 18, 31, 37, 62 and 95 pmol of dermorphin. A group of eight rats was used for each dose. To assess the characteristics of the functional interaction between the supraspinal μ- and δ-receptor agonists, the [D-Ala^2, Glu^4]deltorphin and dermorphin doses were combined in a fixed or variable ratio. Each combination was tested in eight rats. [D-Ala^2, Glu^4]deltorphin (60 nmol, i.c.v.), dermorphin (62 pmol, i.c.v.) and the combinations of 1.2 and 12.4 pmol of dermorphin with 13 nmol of [D-Ala^2, Glu^4]deltorphin were also tested in groups of five rats each, that were pretreated with one of the following μ- or δ-opioid antagonists: naloxone (0.1 mg kg^{-1}, s.c., 20 min before), naloxonazine (10 mg kg^{-1}, i.v., 24 h before), β-funaltrexamine (19 nmol, i.c.v., 24 h before), naltrindole (2 nmol, i.c.v., 90 and 5 min before) and DALCE (10 and 20 nmol, i.c.v., 24 h before). The doses and times we used were those known to obtain the best selectivity and antagonist activity in the rat (Ling *et al.*, 1986; Liu-Chen *et al.*, 1991; Calcagnetti *et al.*, 1990a; Calcagnetti & Holtzman, 1991).

Drugs

[D-Ala^2, Glu^4]deltorphin and dermorphin, were synthesized and purified as previously described (Erspamer *et al.*, 1989). Naloxone (S.A.L.A.R.S. Como, Italy), naltrindole (Research Biochemicals Inc., Natick, MA, U.S.A.) and β-funaltrexamine (Research Biochemicals Inc., Natick, MA, U.S.A.) were dissolved in normal saline; [D-Ala^2, Glu^4]deltorphin and DALCE ([D-Ala^2, Leu^5, Cys^6]enkephalin) (Peninsula Laboratories Europe, Merseyside, England) were dissolved in 10% DMSO (dimethyl sulphoxide), naloxonazine (Research Biochemicals Inc., Natick, MA, U.S.A.) in 0.1% acetic acid. For binding experiments: [^{3}H]-DAMGO ([^{3}H]-[D-Ala^2, MePhe^4, Gly-ol^5]-enkephalin) was purchased from Amersham, U.K.; [^{3}H]-[D-Ala^2, Glu^4]deltorphin and [^{3}H]-naltrindole from NEN Products, Du Pont de Nemours Italiana, Milano, Italy; Gpp[NH]p (5'-guanylylimidodiphosphate) from Sigma, St. Louis, U.S.A.

Data analysis and statistics

Log dose-response curves, each containing at least four doses, were plotted with a nonlinear curve-fitting computer program (PRISM, GraphPad, CA, U.S.A.) and compared with the log dose-response curves of the single agonists. The nonlinear regression program analysed the dose-response curves and calculated the A_{50} values, the curve slopes with 95% confidence limits and the *P* values for slope difference from zero (two-tailed test). Values within square brackets are 95% confidence interval. In variable dose-ratio experiments, for each fixed dose of one of the two agonists a log dose-response curve was constructed by plotting the combined doses of the other agonist against the antinociceptive response obtained with each combination. The time-course of the antinociceptive response was plotted with cubic spline curve fitting. For statistical analysis the CSS: STATISTICA software package (StatSoft, Tulsa, OK, U.S.A.) was used. The Bartlett's test was used for preliminary analysis of the homogeneity of variance. The data were then compared with a two-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test.

Results

Single agonist

Base-line latencies to tail-flick averaged 3.4 ± 0.4 s. Neither i.c.v. saline nor vehicle injection affected latency to tail-flick. Dermorphin induced dose-related increases in the analgesic response (A_{50} = 37.6 [23.4, 58.8] pmol/rat) and was fully efficacious, the maximum achievable response (MPE = 100) having an AUC_{max} of 7950 ± 430 (Figure 1). The analgesic effect became evident 15 min after injection, peaked from 30 to 45 min and lasted for at least 60 to 90 min. [D-Ala^2, Glu^4]-deltorphin had only limited efficacy (Figure 1). Doses lower than 20 nmol never induced a significant antinociceptive response; 60 nmol produced an antinociceptive effect equal to 35 ± 13.8% of dermorphin AUC_{max} and doses up to 90 nmol produced no greater effect (39 ± 14%; AUC = 3100 ± 308). The maximum peak response (83 MPE, 90 nmol) occurred at 15 min and antinociception faded out in 45 min. Twenty minutes after i.c.v. injection in rats, 60 nmol of [D-Ala^2, Glu^4]deltorphin produced a highly significant occupancy of more than 97% of brain δ opioid receptors and an occupancy

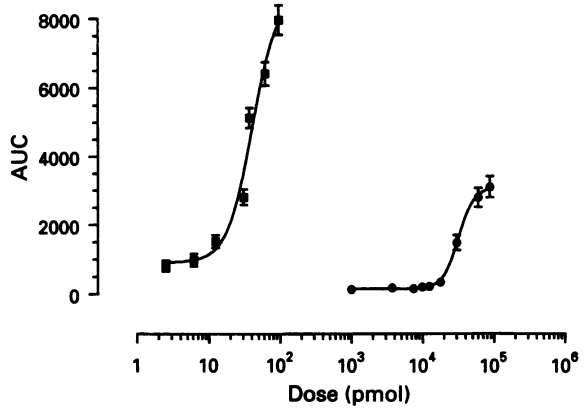

Figure 1 Dose-response curves of the antinociception produced by i.c.v. injection of dermorphin (■) and [D-Ala², Glu⁴]deltorphin (●) in rats (tail-flick test to radiant heat). Data are presented as mean ± s.e.mean for groups of 8 rats.

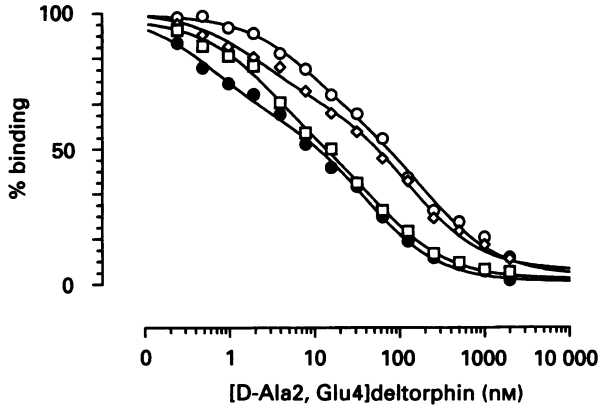

Figure 2 Inhibition of 0.1 nM [³H]-naltrindole binding to rat brain membranes by deltorphin (●), deltorphin plus 10 nM dermorphin (□), deltorphin plus 100 μM Gpp[NH]p (◇), deltorphin plus 100 μM Gpp[NH]p and 10 nM dermorphin (○). Curve comparisons (two way ANOVA): (◇) vs (●), F(1, 64) = 679, $P < 0.0001$; (□) vs (●), F(1, 64) = 59.1, $P < 0.0001$; (○) vs (◇), F(1, 64) = 43.2, $P < 0.0001$; (○) vs (●), F(1, 64) = 1026, $P < 0.0001$.

of about 17% of μ opioid receptors. At the dose of 13 nmol, the peptide occupied about 73% of brain δ opioid receptors but it did not bind to μ opioid receptors at all (Table 1). Dilution and washing of brain membranes whose opioid receptors had been previously equilibrated with 3 nM [³H]-[D-Ala², Glu⁴]deltorphin for 90 min at 35°C, did not significantly modify the specific binding of the ligand. Addition of 10 nmol of [³H]-[D-Ala², Glu⁴]deltorphin to 100 ml of cold (4°C) brain homogenate before the washing and centrifugation steps produced an opioid receptor occupancy of less than 10% B_{max}. These results make it unlikely that homogenization, dilution and washing of brain membranes significantly affected the bound fraction of the drug injected.

Combined μ- and δ-agonists

In vitro *binding* Inhibition of [³H]-naltrindole binding to rat brain membranes by [D-Ala², Glu⁴]deltorphin was best fitted by a two-site model (Figure 2). The addition of 100 μM Gpp(NH)p (a non-hydrolizable form of GTP) to the incubation medium increased by 3.5 times the [D-Ala², Glu⁴]deltorphin K_i values for both sites ('G shift') without affecting B_{max}. Without Gpp(NH)p, the addition of 10 nM dermorphin to the incubation medium increased by 4.5 times the [D-Ala², Glu⁴]deltorphin K_i for the first site (high affinity site) without affecting the K_i for the second site (low affinity site). Gpp(NH)p plus dermophin produced a 15 times increase in the [D-Ala², Glu⁴]deltorphin K_i for the high affinity site and a 5.5 times increase in the K_i for the low affinity site. Thus the 'G shift' of [D-Ala², Glu⁴]deltorphin K_i was significantly greater in the presence of dermorphin. Dermorphin and Gpp(NH)p did not modify the naltrindole affinity for the δ opioid receptor (Table 2).

Antinociception: variable dose-ratio Seven subanalgesic doses of dermorphin (0.1 to 12.4 pmol) were combined with each of four subanalgesic [D-Ala², Glu⁴]deltorphin doses (1.3, 7.4, 9.9 and 13 nmol) to obtain δ:μ dose-ratios ranging from 100 to 13000. When injected i.c.v. in rats, combinations of deltorphin 1.3 nmol with dermorphin doses ranging from 0.74 to 12.4 pmol produced significant antinociceptive responses. Co-administration of 7.4, 9.9 or 13 nmol of [D-Ala², Glu⁴]deltorphin with dermorphin resulted in further significant increases of the AUC values (Figure 3). However, for each [D-Ala², Glu⁴]deltorphin dose tested, the combined doses of dermophin produced the same antinociceptive response. The time-response curve illustrates this better (Figure 4). A combination of the lowest (0.74 pmol) or the highest (12.4 pmol) dose of dermophin with 13 nmol of [D-Ala², Glu⁴]deltorphin gave similar MPE and AUC values. Repeated attempts to obtain a

Table 1 *In vivo* occupancy of brain δ- and μ-opioid receptors by i.c.v. injection of [D-Ala²,Glu⁴]deltorphin (DELT) and naltrinodole (NLT) in rats

Ligand treatment	B_{max} (fmol mg^{-1})	% *change*	K_D (nM)	% *change*
[³H]-DELT				
Saline	68.5 ± 7.5		2.03 ± 0.2	
NLT (2+2 nmol)	0†		0†	
DELT (60 nmol)	2.5 ± 0.5**	−96	6.17 ± 1.0	+204
DELT (13 nmol)	18.5 ± 2.1**	−73	2.19 ± 0.3	–
[³H]-DAMGO				
Saline	97 ± 2		1.16 ± 0.1	
NLT (2+2 nmol)	95 ± 3	−2	1.38 ± 0.1	+19
DELT (60 nmol)	81 ± 2*	−17	1.54 ± 0.2	+33

†The specific binding of [³H]-DELT was minimal, which precluded accurate estimates of receptor density and affinity. Data represent means ± s.e. of five experiments. *$P < 0.05$, **$P < 0.001$ vs. saline.

Table 2 K_i and B_{max} values for inhibition of 0.1 nM [³H]-naltrindole binding to rat brain membranes by various opioid combinations

Inhibitors	K_i (nM)	K_i *ratio*	B_{max} (fmol mg^{-1})	% *sites*
NLT	0.35 ± 0.11		63.0	
NLT + DER	0.29 ± 0.09		66.0	
NLT + Gpp	0.31 ± 0.11		64.0	
DELT				
site I	0.44 ± 0.12	1	25.7	39
site II	28.4 ± 11.3	1	40.3	61
DELT + Gpp				
site I	1.6 ± 0.41*	3.6	22.8	35
site II	91.3 ± 21.2**	3.2	42.3	65
DELT + DER				
site I	2.02 ± 0.48*	4.6	32.3	49
site II	38.3 ± 13.1††	1.4	33.7	51
DELT + DER + Gpp				
site I	6.6 ± 1.58†	15	27.9	41
site II	155 ± 36‡	5.5	40.1	59

NLT = naltrindole; DELT = [D-Ala²,Glu⁴]deltorphin; DER = dermorphin; Gpp = Gpp(NH)p. Tukey's test: *$P < 0.05$ vs site I K_i of DELT; **$P < 0.05$ vs site II K_i of DELT; ††$P > 0.05$ vs site II K_i of DELT; †$P < 0.05$ vs site I K_i of DELT-Gpp; ‡$P > 0.05$ vs site II K_i of DELT-Gpp.

significant antinociceptive response with combinations containing less then 0.74 pmol of dermophin and 13 nmol of [D-Ala^2, Glu^4]deltorphin provided erratic results. A small % (<20%) of rats showed a near 100 MPE antinociceptive response, which lasted few minutes (15–20) and had a small AUC value (about 1500); the remaining animals gave negative results so that the mean AUC value of the group did not differ significantly from that of rats injected with dermorphin alone.

Six subanalgesic doses of deltorphin (1.3 to 18 nmol) were combined with each of five subanalgesic doses of dermorphin (0.74, 1.24, 2.5, 6.2 and 12.4) to obtain δ:μ ratios ranging from 100 to 24000. The combinations containing deltorphin doses ranging from 3.7 to 18 nmol produced significant antinociceptive responses log-related to the δ agonist dose. The resulting dose-response curves of the combinations were all equally and significantly shifted to the left of the deltorphin curve and the maximum achievable antinociceptive response was always obtained (Figure 5).

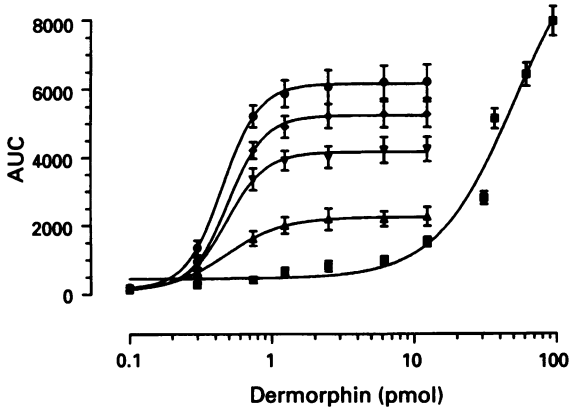

Figure 3 Dose-response curves of the antinociception produced by i.c.v. injection of dermorphin alone (■) and combinations of dermophin with 1.3 (▲), 7.4 (▼), 9.9 (◆) and 13 (●) nmol of [D-Ala^2, Glu^4]deltorphin, in rats (tail-flick test to radiant heat). Data are expressed as mean ± s.e.mean for groups of 8 rats. For the dermorphin dose range of 0.74–13 nmol, two-way ANOVA with post-hoc Tukey's test showed a significant difference in the AUC values between μ-δ combinations and dermorphin alone ($P<0.001$).

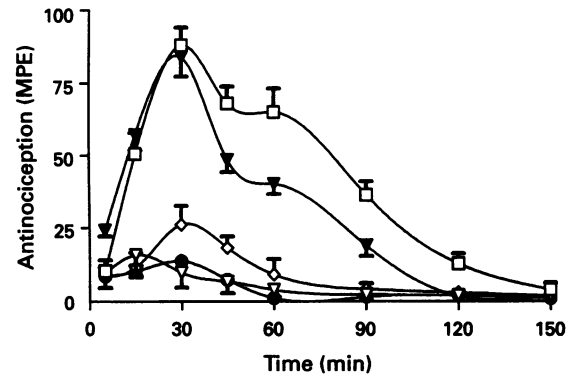

Figure 4 Time-response curves of the antinociception produced by i.c.v. injection of 0.74 (●) and 12.4 pmol (◇) of dermorphin alone, 13 nmol of [D-Ala^2, Glu^4]deltorphin alone (▽) and combinations of 0.74 (▼) or 12.4 pmol (□) of dermorphin with 13 nmol of [D-Ala^2, Glu^4]deltorphin, in rats (tail-flick test to radiant heat). Each point represents the mean ± s.e.mean MPE (maximum achievable response) value for groups of 5 rats. ANOVA with repeated measures showed significant interactions between time and treatment ($P<0.05$) for the following time intervals: 12.4 pmol of dermorphin, 30 and 40 min; 0.74 pmol of dermorphin + 13 nmol of deltorphin and 12.4 pmol of dermorphin + 13 nmol of deltorphin from 15 to 90 min. Tukey's test showed a significant difference in MPE values between the two μ-δ combinations and dermorphin or deltorphin alone ($P<0.001$), but not between the two μ-δ combinations.

Antinociception: fixed dose-ratio When combined with [D-Ala^2, Glu^4]deltorphin in fixed dose-ratios, dermorphin apparently increased from 16 to 64 times in antinociceptive potency (Figure 6). However, because the 95% confidence intervals overlapped, none of the A_{50} values of the four fixed dose-ratio curves were significantly different from each other, but they all differed significantly from the A_{50} of dermorphin alone.

Antagonists

Antagonists were used against dermorphin alone, against [D-Ala^2, Glu^4]deltorphin alone and against two combinations of 0.74 and 12.4 pmol of dermorphin and 13 nmol of [D-Ala^2, Glu^4]deltorphin. Naloxone, naloxonazine and β-funaltrexamine sharply reduced the antinociceptive effect produced by dermorphin (62 pmol, i.c.v.) (Table 3) and by the two opioid combinations (Figure 7).

Surprisingly, the low dose of naloxone (0.1 mg kg^{-1}) and the pretreatment with naloxonazine also antagonized the weak

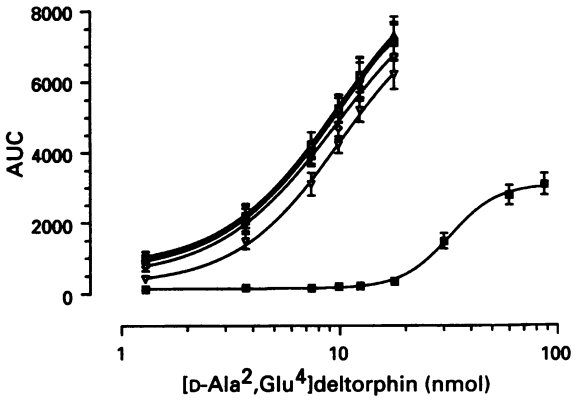

Figure 5 Dose-response curves of the antinociception produced by i.c.v. injection of [D-Ala^2, Glu^4]deltorphin alone (■) and combinations of [D-Ala^2, Glu^4]deltorphin with 0.74 (▽), 1.24 (◇), 2.5 (○), 6.2 (□) and 12.4 pmol (△) of dermorphin in rats (tail-flick test to radiant heat). Data are presented as mean ± s.e.mean for groups of 8 rats. None of the five agonist-combination curves were significantly different from each other, but they all differed significantly ($P<0.001$) from the curve for [D-Ala^2, Glu^4]deltorphin alone.

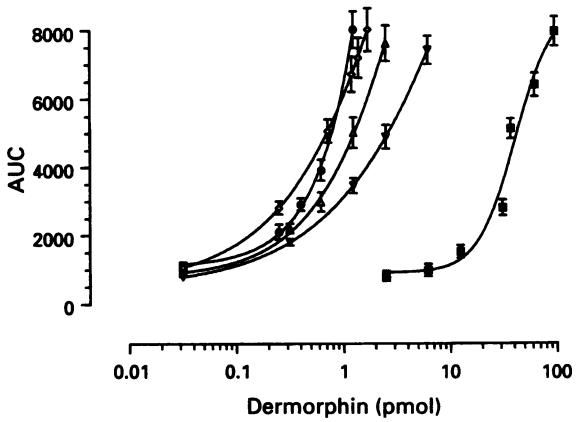

Figure 6 Dose-response curves of the antinociception produced by i.c.v. injection of dermorphin alone (■) and fixed-ratio combinations of dermorphin with [D-Ala^2, Glu^4]deltorphin, in rats (tail-flick test to radiant heat). Dermorphin-[D-Ala^2, Glu^4]deltorphin ratios in the combinations are: (▼) 10^{-3}, (△) 5×10^{-4}, (◇) 2×10^{-4}, (●) 10^{-4}. Each point represents the mean ± s.e.mean AUC value for 5 rats. A_{50} values are: 10^{-3} ratio, 2.42 [1.3, 5.7] pmol; 5×10^{-4} ratio, 1.17 [0.6, 2.2] pmol; 2×10^{-4} ratio, 0.58 [0.16, 2.12] pmol; 10^{-4} ratio, 0.7 [0.39, 1.57] pmol; and dermorphin alone, 37.6 [23.4, 58.8] pmol.

Table 3 Antinociceptive response to [D-Ala^2,Glu^4]deltorphin (DELT) (60 nmol, i.c.v.) and dermorphin (DER) (62 pmol, i.c.v.) in rats pretreated with naloxone (NLX), naltrindole (NLT), naloxonazine (NLXZ), [D-Ala^2,Leu^5,Cys^6]enkephalin (DALCE) and β-funaltrexamine (β-FNA)

	Antinociception	
Treatment	*MPE*	*AUC*
DELT + Saline	75.7 ± 12	2871 ± 280
DELT + NLX (0.1 mg kg^{-1}, s.c.)	14.0 ± 1.7‡	507 ± 97‡
DELT + NLXZ (10 mg kg^{-1}, i.v.)	33.7 ± 2.1‡	1195 ± 333‡
DELT + NLT (2 + 2 nmol, i.c.v.)	88.2 ± 11	3228 ± 409
DELT-DALCE (20 nmol, i.c.v.)	95.2 ± 5	4093 ± 760
DER + saline	75.3 ± 8.5	6154 ± 498
DER + DALCE (20 nmol, i.c.v.)	70.8 ± 9	5695 ± 790
DER + NLX (0.1 mg kg^{-1}, s.c.)	17 ± 5.1†	671 ± 217†
DER + NLXZ (10 mg kg^{-1}, i.v.)	18 ± 4.4‡	741 ± 173†
DER + β-FNA (19 nmol, i.c.v.)	12 ± 4.7†	342 ± 325†

Values are means ± s.e.: ‡$P < 0.05$ compared with DELT-treated rats. †$P < 0.05$ compared with DER treated rats. MPE = maximum achievable response; AUC = area under the curve.

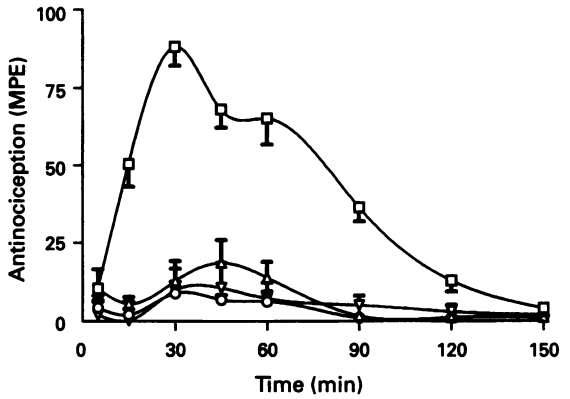

Figure 7 Antagonism by naloxone (△), naloxonazine (○), β-funaltrexamine (▽) pretreatment of the antinociceptive response produced by a combination of 12.4 pmol dermorphin and 13 nmol [D-Ala^2, Glu^4]deltorphin (□), in rats. Each point represents the mean ± s.e.mean AUC value for 5 rats. ANOVA with repeated measures showed significant interactions between time and treatment ($P < 0.001$). Tukey's test showed significant difference in maximum achievable response (MPE) values between the animal groups tested with μ-δ combination alone and those pretreated with opioid antagonists.

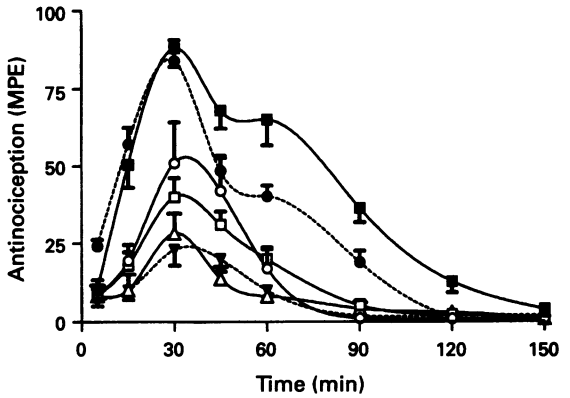

Figure 8 Antagonism by naltrindole (NLT) and [D-Ala^2, Leu^5, Cys^6]enkephalin (DALCE) pretreatment of the antinociceptive response induced by the combinations of 0.74 or 12.4 pmol of dermorphin (DER) with 13 nmol of [D-Ala^2, Glu^4]deltorphin (DELT), in rats (tail-flick test to radiant heat): (●) DER 0.74 pmol + DELT 13 nmol; (■) DER 12.4 pmol + DELT 13 nmol; (▼) NLT 2 + 2 nmol + DER 0.74 pmol + DELT 13 nmol; (□) NLT 2 + 2 nmol + DER 12.4 pmol + DELT 13 nmol; (○) DALCE 20 nmol + DER 12.4 pmol + DELT 13 nmol; (△) DER 12.4 pmol. Each point represents the mean ± s.e.mean AUC value for 5 rats. ANOVA with repeated measures showed a significant difference between the following treatments: (●) vs (▼), $F = 222.59$ (1, 64), $P < 0.0001$; (■) vs (□), $F = 141.32$ (1, 64), $P < 0.0001$; (■) vs (○), $F = 58.70$ (1, 64), $P < 0.0001$.

antinociception produced by the high dose of [D-Ala^2, Glu^4]-deltorphin alone (60 nmol) (Table 3). The δ-selective antagonist naltrindole significantly decreased the antinociceptive effect of the μ-δ combinations (Figure 8). Pretreatment of rats 24 h before testing with 20 nmol of DALCE did not antagonize the antinociceptive effect of dermorphin (Table 3), but it significantly reduced the positive antinociceptive co-operation of the two peptides (Figure 8). Neither naltrindole nor DALCE affected the antinociceptive response produced by 60 nmol of [D-Ala^2, Glu^4]deltorphin alone (Table 3).

Naltrindole injected i.c.v. at the dosage used in the antagonism experiments occupied more than 98% of the brain δ opioid receptors. Naltrindole occupancy of the μ opioid receptors was not significant (Table 1).

Discussion

Evidence of a functional interaction between μ- and δ-opioid agonists comes from studies *in vivo* showing that μ agonists, such as morphine or normorphine, and δ agonists, such as DPDPE or [D-Ala^2, Glu^4]deltorphin, interact to enhance their antinociceptive potency and efficacy. Yet other studies have failed to demonstrate a co-operation between these δ agonists and other μ-selective agonists, such as sufentanil, PLO17 and DAMGO. Because δ agonists do not produce antinociception in rats when the noxious stimulus for analgesic testing is radiant heat, the μ-δ interaction has not been previously studied using the classical tail-flick test of D'Amour and Smith (1941). In mice, however, with radiant heat as the noxious stimulus, Lee *et al.* (1980) have described the modulatory effects of enkephalins on morphine antinociception, even though it is virtually impossible, in this test, to demonstrate direct antinociceptive effects of these peptides.

Our present findings clearly show co-operation between the μ-selective agonist dermorphin and the δ-selective agonist [D-Ala^2, Glu^4]deltorphin, both *in vitro* and *in vivo*. *In vitro* studies on rat brain membranes demonstrated that the μ-agonist dermorphin increased the K_i value of δ-agonist for the high affinity site of [^{3}H]-naltrindole binding without affecting the B_{max} and the K_i of the radioactive ligand. It also enhanced the increase in K_i value of [D-Ala^2, Glu^4]deltorphin produced by 100 μM Gpp[NH]p and thus co-operated in the activation of

the high affinity site of δ receptors. *In vivo*, a positive antinociceptive co-operation between the μ selective agonist dermorphin and the δ-selective agonist [D-Ala^2, Glu^4]deltorphin, was evident in the rat tail-flick response to radiant heat. Even though [D-Ala^2, Glu^4]deltorphin acts as an extremely weak partial agonist and dermorphin behaves as a full agonist in this test, injecting i.c.v. several variable or fixed-ratio combinations of subanalgesic doses of the two peptides generated full dose-effect curves. The antinociceptive response was linearly related to the log dose of the δ-agonist contained in the combination. For each combined [D-Ala^2, Glu^4]deltorphin dose, dermorphin doses lower than 0.74 pmol never resulted in significant antinociception, whereas doses of the μ-agonist ranging from 0.74 to 12 pmol all potentiated the antinociceptive response to the same degree. A possible, but speculative explanation of this observation might be that the occupancy of a minimum number of μ-receptors is essential for effective transduction of the δ-message into an antinociceptive response. The presence of subanalgesic doses of the μ-agonist in the mixture also increased the efficacy of the δ-agonist, so that the highest combined dose (13 nmol) of [D-Ala^2, Glu^4]deltorphin produced the maximum achievable response (although determination of the efficacy depends on an artificially determined maximum effect). The reversible and non-equilibrium μ-antagonists completely blocked the antinociceptive responses to all tested doses of the μ-agonist and to the combinations of the two agonists. Naloxone and, to a lesser extent, naloxonazine, also antagonized the antinociceptive response evoked by 60 nmol of [D-Ala^2, Glu^4]deltorphin alone. Pretreatment with reversible and non-equlibrium δ-antagonists reduced the response of the combinations to a value near to that produced by the μ-agonist alone, but did not antagonize the antinociceptive response produced by dermorphin or by 60 nmol of [D-Ala^2, Glu^4]-deltorphin alone. However, because *in vivo* binding to opioid receptors showed that this [D-Ala^2, Glu^4]deltorphin dose occupied about 17% of the brain μ-opioid receptors its antinociceptive effect is presumably μ-receptor mediated. This conclusion concurs with findings obtained by Raffa *et al.* (1992) in the CXBK mouse strain, which is deficient in μ-opioid receptors. In these mice i.c.v. administration of [D-Ala^2, Glu^4]deltorphin did not produce antinociception. To date, only one δ opioid receptor has been cloned, but pharmacological data suggest that [D-Ala^2, Glu^4]deltorphin activates a δ subtype (δ_2) different from that preferred by DPDPE (δ_1) (Sofuoglu *et al.*, 1991; Jiang *et al.*, 1991; Mattia *et al.*, 1991). Our results indicate that the δ-opioid receptor subtype participating in the μ-δ interaction, is the δ_2 receptor subtype. In our experiments with dermorphin-DPDPE combinations we observed evidence of increased antinociception only when putative δ_1 agonist doses that were *per se* analgesic were combined with the μ-agonist (data not shown). Thus, because we were unable to exclude the possibility that dermorphin-DPDPE antinociception was produced by activation of μ-opioid receptors, we considered this drug combination not adequate to demonstrate a μ-δ_1 interaction, at least in the rat tail-flick test.

In conclusion, our results indicate that subanalgesic doses of [D-Ala^2, Glu^4]deltorphin (1.3 to 13 nmol) combined with dermorphin, acted on δ-opioid receptors to produce antinociception. In this interaction, the μ-opioid agonist appears to modulate positively an antinociceptive response dose-dependently evoked by the δ-opioid agonist. Our present results cannot definitively answer the question whether this μ-δ antinociceptive co-operation involves interactions between different brain opioid pathways or between μ and δ receptors at the same cellular level. Rossi *et al.* (1994) demonstrated μ-δ positive antinociceptive cooperation between the periacqueductal gray (PAG) and the rostral ventral medulla (RVM) of rats when DAMGO was co-administered into one region and [D-Ala^2, Glu^4]deltorphin into the other. When the two opioid agonists were injected in the same region additive effects were recorded. Rossi *et al.* conclude that μ-δ positive antinociceptive co-operation in the rat appears to involve pathway interactions rather than receptor interactions. Obviously pathway interactions can operate also in our experimental conditions, but they seem unlikely to be the only kind of interaction intervening in the antinociceptive co-operation between dermorphin and [D-Ala^2, Glu^4]deltorphin. Firstly, Rossi *et al.* reported that combinations of DAMGO and [D-Ala^2, Glu^4]deltorphin in the PAG were not synergistic when peak antinociceptive responses were considered, but showed superadditive effects when antinociception was measured by AUC. Secondly, the PAG/RVM multiplicative interactions also operate for μ-opioid agonists alone but they do not occur when δ-opioid agonists alone are injected into the two regions. Thus in our experimental conditions, where combinations of μ- and δ-agonists given i.c.v. act simultaneously at PAG and RVM and the antinociceptive response was measured by AUC, pathway interactions alone are not sufficient to explain the synergistic antinociception. Though the tail-flick test in the rat is extremely sensitive to spinal antinociceptive activity, previous studies have demonstrated that the spinal antinociceptive threshold for dermorphin and [D-Ala^2, Glu^4]deltorphin is in the range of 2–10 ng and 2–5 μg, respectively (Improta & Broccardo, 1992), which is within the range of doses given by i.c.v. route in the present work. However, we can exclude the possibility that either or both ligands could have acted at a spinal level because evidence exists (Dauge *et al.*, 1987) that tritiated enkephalin analogues injected into the lateral ventricles of rats remain localized in the brain and less than 1% of the amount injected appears in the spinal cord. Finally, our binding studies demonstrated that low concentrations of the μ-agonist positively co-operated with the δ-agonist in the activation of δ-opioid receptors. The increase in the [D-Ala^2, Glu^4]deltorphin K_i produced by dermorphin in the absence and in the presence of Gpp[NH]p may explain the increase in antinociceptive potency and efficacy observed *in vivo* with μ-δ agonist mixture and indicates that μ-δ co-operation involves receptor interactions at the cellular level.

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References

ADAMS, J.U., TALLARIDA, R.J., GELLER, E.B. & ADLER, M.W. (1993). Isobolographic superadditivity between delta and mu opioid agonists in the rat depends on the ratio of compounds, the mu agonist and the analgesic assay used. *J. Pharmacol. Exp. Ther.*, **266**, 1261–1267.

CALCAGNETTI, D.J., BOWEN, W.D. & HOLTZMAN, S.G. (1990a). Stress-induced tolerance to delta receptor agonist DPDPE and selectivity for the irreversible δ-ligand DALCE. *Brain Res.*, **509**, 205–212.

GALCAGNETTI, D.J., FLEETWOOD, S.W. & HOLTZMAN, S.G. (1990b). Pharmacological profile of the potentiation of opioid analgesia by restraint stress. *Pharmacol. Biochem. Behav.*, **37**, 193–199.

CALCAGNETTI, D.J. & HOLTZMAN, S.G. (1991). Delta opioid antagonist, naltrindole, selectively blocks analgesia induced by DPDPE but not DAGO or morphine. *Pharmacol. Biochem. Behav.*, **38**, 185–190.

CHA, X.Y., XU, H., CALDERON, S.N., SMITH, L.E., RICE, K.C., PORRECA, F. & ROTHMAN, R.B. (1994). BW373U86, its enantiomers, and related compounds: Interactions of non-peptide delta agonist at multiple delta (cx) binding sites in rat brain. *Reg. Peptides*, **54**, 45–46.

D'AMOUR, F.E. & SMITH, D.L. (1941). Method for determining loss of pain sensation. *J. Pharmacol. Exp. Ther.*, **72**, 74–79.

DAUGE, V., PETIT, F., ROSSIGNOL, P. & ROQUES, B.P. (1987). Use of mu and delta opioid peptides of various selectivity give further evidence of specific involvement of mu opioid receptors in supraspinal analgesia (tail-flick test). *Eur. J. Pharmacol.* **141**, 171–178.

ERSPAMER, V., MELCHIORRI, P., FALCONIERI ERSPAMER, G., NEGRI, L., CORSI, R., SEVERINI, C., BARRA, D., SIMMACO, M. & KREIL, G. (1989). Deltorphins: a family of naturally occurring peptides with high affinity and selectivity for δ opioid binding sites. *Proc. Natl. Acad. Sci. U.S.A.*, **86**, 5188–5192.

HEYMAN, J.S. (1987). Modulation of supraspinal μ opioid inhibition of gastrointestinal propulsion by δ agonists in the mouse. *Gastroenterology*, **92**, P1801.

HEYMAN, J.S., MOSBERG, H.I. & PORRECA, F. (1987). Species dependent analgesic effects of [D-Pen^2,D-Pen^5]enkephalin (DPDPE). *Pharmacologist*, **29**, 106–109.

HEYMAN, J.S., VAUGHT, J.L., MOSBERG, H.I., HAASETH, R.C. & PORRECA, F. (1989). Modulation of μ-mediated antinociception by δ agonists in mouse. Selective potentiation of morphine and normorphine by [D-Pen^2,D-Pen^5]enkephalin. *Eur. J. Pharmacol.*, **165**, 1–10.

HOLADAY, J.W. & D'AMATO, R.J. (1983). Multiple opioid receptors: evidence for μ-δ binding site interactions in endotoxic shock. *Life Sci.*, **33**, 703–706.

HORAN, P., TALLARIDA, R.J., HAASETH, R.C., MATSUNAGA, T.O., HRUBY, V.J. & PORRECA, F. (1992) Antinociceptive interactions of opioid delta receptor agonists with morphine in mice: supra- and sub-additivity. *Life Sci.*, **50**, 1535–1541.

IMPROTA, G. & BROCCARDO, M. (1992). Spinal antinociceptive effects of [D-Ala^2]deltorphin II, a novel and highly selective delta-opioid receptor agonist. *Peptides*, **13**, 1126–1129.

KAMEI, J., IWAMOTO, Y., SUZUKI, T., NAGASE, H., MISAWA, M. & KASUYA, Y. (1993). Differential modulation of μ-opioid receptor-mediated antitussive activity by δ-opioid-receptor agonists in mice. *Eur. J. Pharmacol.*, **234**, 117–120.

JIANG, Q., MOSBERG, H.I. & PORRECA, F. (1990). Modulation of the potency and efficacy of mu-mediated antinociception by delta agonists in the mouse. *J. Pharmacol. Exp. Ther.*, **254**, 683–689.

JIANG, Q., TAKEMORI, A.E., SULTANA, M., PORTOGHESE, P.S., BOWEN, W.D., MOSBERG, H.I. & PORRECA, F. (1991). Differential antagonism of opioid delta antinociception by [D-Ala^2, Leu^5, Cys^6]enkephalin and naltrindole 5'-isothiocyanate: evidence for delta-receptor subtypes. *J. Pharmacol. Exp. Ther.*, **257**, 1069–1075.

LEE, N.M., LEYBIN, L., CHANG, J.K. & LOH, H.H. (1980). Opiate and peptide interaction: Effect of enkephalins on morphine analgesia. *Eur. J. Pharmacol.*, **68**, 181–185.

LING, G.S.F., SIMANTOV, R., CLARK, J.A. & PASTERNAK, G.W. (1986). Naloxonazine actions *in vivo*. *Eur. J. Pharmacol.*, **129**, 33–38.

MATTIA, A., VANDERAH, T., MOSBERG, H.I. & PORRECA, F. (1991). Lack of antinociceptive cross tolerance between [D-Pen^2,D-Pen^5]enkephalin and [Ala^2]deltorphin II in mice: Evidence for delta receptor subtypes. *J. Pharmacol. Exp. Ther.*, **258**, 583–587.

MELCHIORRI, P., MARITATI, M., NEGRI, L. & ERSPAMER, V. (1992). Long-term sensitization to the activation of cerebral delta-opioid receptors by [D-Ala^2]deltorphin II in rats exposed to morphine. *Proc. Natl. Acad. Sci. U.S.A.*, **89**, 3696–3700.

NEGRI, L., LATTANZI, R. & MELCHIORRI, P. (1995). [Lys^7]dermorphin, a naturally occurring peptide with high affinity for μ-opioid receptors, produces antinociception by peripheral administration. *Br. J. Pharmacol.*, **114**, 57–66.

NEGRI, L., NOVIELLO, L., NOVIELLO, V. & ANGELUCCI, F. (1991). Behavioural effects of deltorphins in rats. *Eur. J. Pharmacol.*, **209**, 163–168.

PORRECA, F., HEYMAN, J.S., MOSBERG, H.I., OMNAAS, J.R. & VAUGHT, J.L. (1987). Role of μ and δ receptors in the supraspinal and spinal analgesic effects of [D-Pen^2,D-Pen^5]enkephalin in the mouse. *J. Pharmacol. Exp. Ther.*, **241**, 393–399.

PORRECA, F., TAKEMORI, A.E., SULTANA, M., PORTOGHESE, P.S., BOWEN, W.D. & MOSBERG, H.I. (1992). Modulation of mu-mediated antinociception in the mouse involves opioid delta-2 receptors. *J. Pharmacol. Exp. Ther.*, **263**, 147–152.

RAFFA, R.B., MARTINEZ, R.P. & PORRECA, F. (1992). Lack of antinociceptive efficacy of intracerebroventricular [D-Ala^2, Glu^4]deltorphin, but not [D-Pen^2,D-Pen^5]enkephalin, in the μ-opioid-receptor deficient CXBK mouse. *Eur. J. Pharmacol.*, **216**, 453–456.

ROSSI, C.G., PASTERNAK, G.W. & BODNAR, R.J. (1994). μ and δ opioid synergy between the periaqueductal gray and the rostroventral medulla. *Brain Res.*, **665**, 85–93.

ROTHMAN, R.B., LONG, J.B., BYKOV, V., JACOBSON, A.E., RICE, K.C. & HOLADY, J.W. (1988). β-FNA binds irreversibly to the opiate receptor complex: *In vivo* and *in vitro* evidence. *J. Pharmacol. Exp. Ther.*, **247**, 405–416.

ROTHMAN, R.B. & WESTFALL, T.C. (1982a). Morphine allosterically modulates the binding of [^{3}H]leucine to a particulate fraction of the rat brain. *Mol. Pharmacol.*, **21**, 538–547.

ROTHMAN, R.B. & WESTFALL, T.C. (1982b). Allosteric coupling between morphine and enkephalin receptors *in vitro*. *Mol. Pharmacol.*, **21**, 548–577.

SHELDON, R.J., NUNAN, L. & PORRECA, F. (1989). Differential modulation by [D-Pen^2,D-Pen^5] enkephalin and dynorphin A-(1-17) of the inhibitory bladder motility effects of selected μ agonists *in vivo*. *J. Pharmacol. Exp. Ther.*, **249**, 462–469.

SOFUOGLU, M., PORTOGHESE, P.S. & TAKEMORI, A.E. (1991). Differential antagonism of delta opioid agonists by naltrindole and its benzofuran analog (NTB) in mice: Evidence for delta opioid receptor subtypes. *J. Pharmacol. Exp. Ther.*, **257**, 676–680.

STAMIDIS, H. & YOUNG, G.A. (1992). Mu-delta opioid interactions I: the delta peptide, DPDPE, increases morphine-induced EEG and EEG spectral power. *Peptides*, **13**, 749–753.

VAUGHT, J.L. & TAKEMORI, A.E. (1979). Differential effect of leucine and methionine enkephalin on morphine-induced analgesia, acute tolerance and dependence. *J. Pharmacol. Exp. Ther.*, **208**, 86–90.

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