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Use of selective antagonists and antisense oligonucleotides to evaluate the mechanisms of BUBU antinociception

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Abstract

Evidence suggests that the antinociceptive effects of selective δ-opioid receptor agonists may involve an activation of the μ-receptor in some experimental conditions. The aim of this study was to clarify the receptors involved in the antinociceptive responses of the selective and systemically active δ-opioid receptor agonist Tyr-D-Ser-(*O-tert*-butyl)-Gly-Phe-Leu-Thr-(*O-tert*-butyl) (BUBU). The antinociception induced by systemic (i.v.) or central (i.c.v.) administration of BUBU was measured in the hot plate (jumping and paw lick latencies) and tail immersion tests in mice. In both tests, the responses were more intense when BUBU was administered by central route. The pre-treatment with the μ-opioid receptor antagonist cyprodime blocked the effects induced by central BUBU in the hot plate and tail immersion tests. The δ-opioid receptor antagonist naltrindole had no effect on BUBU-induced antinociception in the hot plate but decreased BUBU responses in the tail immersion test. Further evidence for this dual receptor action of BUBU was demonstrated by using antisense oligodeoxynucleotides. Thus, a reduction in central BUBU-induced antinociception was observed in the tail immersion test after the administration of antisense probes that selectively blocked the expression of μ- or δ-opioid receptors. These findings clearly indicate using a dual pharmacological and molecular approach that BUBU mediates its antinociceptive effects via activation of both μ- and δ-opioid receptors.

Keywords: BUBU; δ-Opioid receptor; Cyprodime; Naltrindole; Antisense oligonucleotide; Antinociception

1. Introduction

Extensive studies using pharmacological, biochemical and molecular techniques have led to the identification of three main opioid receptor types, namely the μ-, δ- and κ-receptor (see Reisine, 1995). Activation of opioid receptors has been shown to mediate a variety of pharmacological effects, some of them related to their therapeutic actions such as analgesia (Ward and Takemori, 1983) and other unwanted effects, such as dependence (Cowan et al., 1988). The μ-Opioid receptor has been shown to mediate the main pharmacological responses of opioids including their unwanted effects (Matthes et al., 1996). Therefore, the development of selective agonists of other opioid receptors, such as δ-opioid receptor, could be one of the possible strategies to decrease the side-effects of opioids. Thus, activation of the δ-opioid receptor by selective agonists produces antinociceptive effects but only weak physical dependence in rodents (Cowan et al., 1988; Maldonado et al., 1990).

Initial attempts to develop ligands with high selectivity for the δ-opioid receptor led to the synthesis of cyclic peptides derived from the endogenous enkephalins, like

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[D-Pen^2, D-Pen^5]enkephalin (DPDPE) (Mosberg et al., 1983). Peptides with a greater δ-opioid receptor selectivity were isolated from the skin of frogs, such as [D-Ala^2]deltorphin II (Kriel et al., 1989). Due to the low ability of these peptide-derived agonists to cross the blood–brain barrier, central administration is required to obtain most of their pharmacological responses, which represents a serious limitation. However, this problem was improved with the development of Tyr-D-Ser-(*O-tert*-butyl)-Gly-Phe-Leu-Thr-(*O-tert*-butyl) (BUBU) (Gacel et al., 1988) a highly selective δ-opioid receptor peptide-derived agonist able to cross the blood–brain barrier (Delay-Goyet et al., 1988) that produces antinociception after systemic administration (Delay-Goyet et al., 1991).

In spite of the high relative selectivity for the δ-opioid receptor shown in vitro by these selective opioid agonists, several findings suggest that some of their antinociceptive effects in vivo may be partly due to the activation of μ-opioid receptors (Chaillet et al., 1984). Thus, antinociceptive responses induced by central administration of peptide-derived δ-opioid receptor selective agonists appear to be blocked by μ-opioid receptor antagonists, whereas δ-opioid receptor antagonists were unable to reverse these responses under some experimental conditions (Chaillet et al., 1984; Cowan et al., 1985; Shook et al., 1987; Baamonde et al., 1991; Bilsky et al., 1994; Shah et al., 1994; Negri et al., 1996).

Another strategy more recently developed to study the opioid system in vivo consists in impairing receptor functions by the use of antisense oligodeoxynucleotides (Wahlestedt et al., 1993; Zhang and Creese, 1993). This strategy has been previously shown to be a validated approach in vitro to inhibit mRNA translation into functional proteins (Cáceres and Kosik, 1990; Holopaien and Wodjcik, 1993; Wagner 1994). Antisense oligodeoxynucleotides to opioid receptors were reported to selectively block the antinociceptive effects of μ-opioid receptor agonists in rats (Chen et al., 1995; Rossi et al., 1997a), the spinal (Standifer et al., 1994; Tseng et al., 1994; Rossi et al., 1997b) and supraspinal (Lai et al., 1994; Bilsky et al., 1996; Rossi et al., 1997b; Sánchez-Blazquez et al., 1997) antinociception induced by several δ-opioid receptor agonists, and the antinociceptive actions exerted by the κ-opioid receptor agonist *trans*-3,4-dichloro-*N*-methyl-1-*N*-1-pyrrolidinyl-(cyclohexyl)-benznecetamide (U-50,488H) in mice (Chien et al., 1994).

The aim of this study was therefore to clarify the opioid receptors involved in BUBU-induced antinociception through both pharmacological (selective antagonists) and molecular techniques (antisense oligodeoxynucleotides). For this purpose, BUBU was firstly co-administered with the selective opioid receptor antagonists cyprodime (μ) and naltrindole (δ). In a second experiment, antisense oligodeoxynucleotides selective for the mRNA involved in the expression of the μ- and separately the δ-opioid receptor were employed to further elucidate their involvement in BUBU antinociception. This study also serves to enable comparison of the effects of antisense oligodeoxynucleotides that cause a knockdown of δ- and μ-opioid receptors with those of the administration of selective antagonists for these receptors.

2. Methods

2.1. Animals

Albino male CD-1 mice (Charles River, France) weighing 20–25 g were housed in cages in groups of five and maintained at a controlled temperature of 21 ± 1°C. The mice were given access to food and water ad libitum. Lighting was maintained at 12-h cycles (on at 0800 h and off at 2000 h). Mice were housed and used strictly in accordance with the guidelines of the European Community about Care and Use of Laboratory Animals, and approved by the Local Ethical Committees.

2.2. Hot plate test

Antinociceptive responses were measured firstly using the water heated hot plate procedure, as described previously (Eddy and Leimbach, 1953). The plate was heated to 52 ± 0.5°C and enclosed by a plexiglass cylinder (diameter 16.5 cm, height 16 cm). Mice were placed individually on the plate and the latency to the first paw lick and first jump event was measured with a cut off time of 30 and 180 s, respectively, to prevent tissue damage.

2.3. Tail immersion assay

Mice were gently placed in a restrainer cylinder for use in the tail immersion assay, as described previously (Janssen et al., 1963). The water used for immersing the tail was maintained at a constant temperature of 50 ± 0.5°C. The time to withdraw the tail from the bath was registered, with a cut off latency of 10 s to prevent tissue damage.

2.4. Synthesis of oligodeoxynucleotides

Synthetic end-capped phosphorothioate antisense oligodeoxynucleotides were prepared by solid phase phosphoramidite chemistry (Matteucci and Caruthers, 1981) using a CODER 300 DNA synthesizer (DuPont, Wilmington, DE, USA) at the 1-μmol scale. The introduction of phosphorothioate linkages was achieved by tetraethylthiuram disulfide sulfurization (Vu and Hirschbein, 1991). Crude oligodeoxynucleotides were purified by conventional reverse-phase chromatography through a 5-$\mu m/C_{18}$ column (Spherisorb ODS-2, 150×4.6 mm^2) using 0.1 M trieth-

ylammonium acetate (pH 7.0) and acetonitrile as mobile phase. The eluted oligodeoxynucleotides were then desiccated (Speed Vac Plus Savant, Farmingdale, NY, USA) and stored at −20°C until use. Sequences were as follows: antisense oligodeoxynucleotide-μ_{16-32} 5′-C*T*GATGTTCCCTGGG*C*C-3′ (Sánchez-Blazquez et al., 1997) a 17 base oligodeoxynucleotide directed to nucleotides 16–32 of mouse μ-opioid receptor cDNA (Min et al., 1994), antisense oligodeoxynucleotide-δ_{7-26} 5′-G*C*ACGGGCAGAGGGCACC*A*G-3′ directed to nucleotides 7–26 of the murine δ-opioid receptor gene sequence (Evans et al., 1992; Kieffer et al., 1992) and identical to that previously used (Lai et al., 1994; Bilsky et al., 1996; Sánchez-Blazquez et al., 1997), and antisense oligodeoxynucleotide-δ_{29-46} 5′-A*G*AGGGCAC-CAGCTCC*A*T-3′ (Standifer et al., 1994; Sánchez-Blazquez et al., 1997) directed to nucleotides 29–46 of the murine δ-opioid receptor gene sequence. A random sequence of oligodeoxynucleotide with the following sequence 5′-C*C*CTTATTTACTACTTTC*G*C-3′ served as a control (Sánchez-Blazquez et al., 1995, 1997). This random sequence has been clearly reported to have all the characteristics of an appropriate control since it displayed no homology to any other relevant cloned protein (GeneBank database), did not modify the levels of μ- or δ-opioid receptors (Sánchez-Blazquez et al., 1997) and did not alter antinociceptive responses induced by several opioid agonists (Sánchez-Blazquez et al., 1995, 1997).

2.5. Chemicals and administration procedures

BUBU (Gacel et al., 1988), naltrindole (Portoghese et al., 1988) and cyprodime (Schmidhammer et al., 1989) were synthesised as previously described. Naltrindole (2.5 mg/kg) and cyprodime (10 mg/kg) were dissolved in saline and injected by s.c. route 20 and 15 min before BUBU injections, respectively. BUBU (lysine salt) was dissolved in distilled water and injected either i.c.v. (Haley and McCormick, 1957) or i.v. through the tail vein, both 10 min before nociceptive measurements.

The doses and pre-treatment times of naltrindole and cyprodime were selected as they have been shown to produce strong antagonism of the antinociceptive responses induced in different models of pain by selective δ- (Sofuoglu et al., 1991; Kalso et al., 1992; Lee et al., 1994) and μ- (Schmidhammer et al., 1989, 1990a,b) opioid receptor agonists, respectively. Furthermore, at these doses, naltrindole (2.5 mg/kg) and cyprodime (10 mg/kg) have also been demonstrated to be selective for their respective target opioid receptors. Higher doses of these antagonists were not used in order to avoid a cross-reactivity with other opioid receptors (Baamonde et al., 1991) and the possible intrinsic antinociceptive effects of naltrindole (Jackson et al., 1989).

2.6. Experimental sequence

2.6.1. Experiment 1: dose–responses curves of BUBU

Antinociceptive responses of BUBU were evaluated in the hot plate and tail immersion tests. BUBU (0.25, 0.5 and 1.0 μg) and saline were administered in a volume of 5 μl for the i.c.v. route. For the systemic i.v. administrations, BUBU (7.5, 15 and 30 mg/kg) and saline were injected in a volume of 0.1 ml per 10 g of body weight. Different mice were used for each group ($n = 13–15$ per group).

2.6.2. Experiment 2: effects of selective opioid receptor antagonists on BUBU-induced antinociception

The most effective route of administration (i.c.v.) and dose (1.0 μg) of BUBU were selected for antagonism studies in the hot plate and tail immersion tests. For this purpose, animals receiving BUBU (1 μg) by i.c.v. route were pre-treated with either saline (s.c.), cyprodime (10 mg/kg, s.c.) or naltrindole (2.5 mg/kg, s.c.) in a volume of 0.1 ml per 10 g of body weight. Vehicle treated controls (i.c.v. and i.v.) were included to measure any nociceptive consequences of the procedure or of the antagonists on their own. Different mice were used for each group ($n =$ 13–18 per group).

2.6.3. Experiment 3: effects of antisense oligodeoxynucleotides on BUBU-induced antinociception

Oligodeoxynucleotide solutions were made up in the appropriate volume of sterile water immediately prior to use. Animals received either the vehicle (control), the random-oligodeoxynucleotide or the antisense oligodeoxynucleotides. These solutions were injected into the right lateral ventricle. Subsequent administrations were performed on the same side. Each oligodeoxynucleotide treatment was performed on a distinct group of 15–20 mice using the following schedule: 1 nmol was administered on days 1 and 2, 2 nmol were administered on days 3 and 4, and 3 nmol were administered on day 5. An additional naive group of mice not receiving any treatment during the first 5 days was included in order to evaluate any intrinsic effects of the experimental procedure. On day 6, BUBU (1 μg/5 μl) or saline (5 μl) were injected (i.c.v.) and the antinociceptive activity evaluated 10 min later in the tail immersion test. An interval of 24 h was selected between oligodeoxynucleotide administrations to minimize the neurotoxic damage (Chiasson et al., 1994).

2.7. Statistical analysis

Antinociceptive data were calculated as the percentage of maximal possible effect (% M.P.E.) using the following formula: (test latency − control mean latency)/(cut-off time − control mean latency) × 100. Statistical significance was determined by one-way analysis of variance (ANOVA) followed by the Student–Newman–Keul's test. The level of significance was set at $P < 0.05$.

3. Results

3.1. Dose–response curves of BUBU

Overall analysis of the i.c.v. data showed significant effects of BUBU administration in the hot plate test for jumping ($F(3,54) = 31.663$, $P < 0.001$) and paw lick latencies ($F(3,54) = 9.236$, $P < 0.001$), and in the tail immersion test ($F(3,54) = 5.644$, $P < 0.01$). Individual comparisons revealed a significant effect of i.c.v. administration of BUBU at the doses of 0.5 ($P < 0.01$) and 1 μg ($P < 0.01$) on jumping and at the dose of 1 μg on paw lick latency ($P < 0.01$) and tail immersion test ($P < 0.01$) (Fig. 1A).

After systemic (i.v.) administration of BUBU, one-way ANOVA showed significant effects in the jumping ($F(3,53) = 5.598$, $P < 0.01$) and paw lick latencies ($F(3,53) = 3.203$, $P < 0.05$) of the hot plate test, and in the tail immersion test ($F(3,53) = 5.959$, $P < 0.001$). However, weaker antinociceptive responses were found when using this route of administration. Thus, individual comparisons showed significant responses in the hot plate test for jumping ($P < 0.01$) and paw lick latencies ($P < 0.05$) and in the tail immersion test ($P < 0.01$) only when BUBU was administered at the highest dose (30 mg/kg) (Fig. 1B).

Taking into account these results, the most effective route of administration (i.c.v.) and dose (1 μg) of BUBU were choosen for the studies with selective opioid receptor antagonists and antisense oligodeoxynucleotides.

3.2. Effects of selective opioid receptor antagonists on BUBU-induced antinociception

Overall analysis showed significant antinociceptive effects in the antagonist experiments in jumping ($F(5,73) = 56.483$, $P < 0.001$) and paw lick latencies ($F(5,73) = 12.478$, $P < 0.001$) of the hot plate test and in the tail immersion test ($F(5,73) = 6.650$, $P < 0.001$). I.c.v. administration of BUBU (1 μg) produced significant antinociceptive responses in saline pre-treated animals in jumping ($P < 0.01$) and paw lick latencies ($P < 0.01$) in the hot plate test as well as in the tail immersion test ($P < 0.01$; Fig. 2), in agreement with the previous experiment (Fig. 1). Neither naltrindole (2.5 mg/kg, s.c.) nor cyprodime (10 mg/kg, s.c.) had any intrinsic effect on basal nociceptive responses when compared to the saline pre-treated group in any of the nociceptive parameters. Pre-treatment with naltrindole failed to attenuate the antinociceptive effects of BUBU in the hot plate test for jumping and paw lick responses. In the tail immersion test, naltrindole significantly ($P < 0.05$) attenuated the antinociception induced by BUBU administration with values not significantly elevated when comparing this group with saline vehicle pre-treated controls. Cyprodime administration completely blocked the antinociceptive responses induced

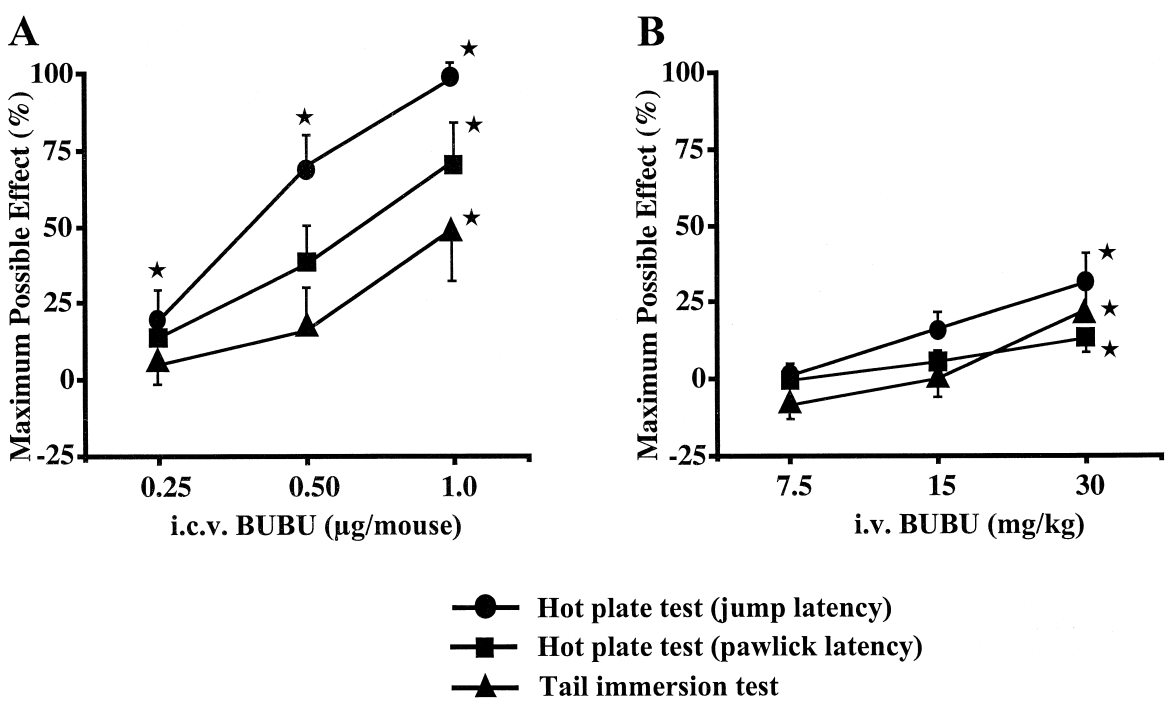

Fig. 1. Antinociceptive responses induced in the tail immersion and hot-plate tests (paw lick and jumping latencies) in mice after administration of BUBU. (A) I.c.v. BUBU administration (0.25, 0.5 and 1 μg). (B) I.v. BUBU administration (7.5, 15 and 30 mg/kg). Antinociception is expressed as a percentage of the maximum possible effect. Values are the mean ± S.E.M. from groups of 13–15 mice each. ★$P < 0.05$ vs. respective control (Newman–Keul's test).

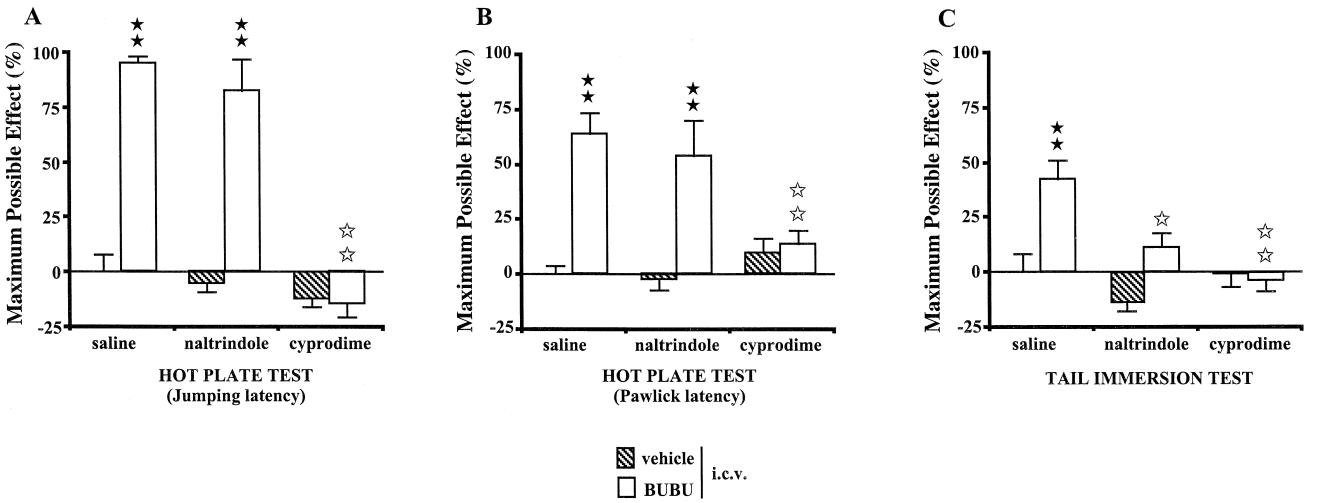

Fig. 2. Effects of selective μ-(cyprodime) and δ-(naltrindole) opioid receptor antagonists on the antinociception induced by central administration of BUBU (1 μg, i.c.v.). (A) Jumping latency in the hot plate test, (B) Paw lick latency in the hot plate test, (C) Tail immersion test. Saline, naltrindole (2.5 mg/kg, s.c.) or cyprodime (10 mg/kg, s.c.) pre-treatments were administered to both vehicle and BUBU groups as indicated on the horizontal axis. Antinociception is expressed as a percentage of the maximum possible effect. Values are the mean ± S.E.M. from groups of 13–18 mice each. ★$P < 0.05$, ★★$P < 0.01$, vs. respective saline control. ☆$P < 0.05$, ☆☆$P < 0.01$, vs. respective BUBU + saline treated group (Newman–Keul's test).

by BUBU in all the parameters with significant decreases in antinocicepetive responses vs. BUBU-treated controls for jumping ($P < 0.01$) and paw lick latencies ($P < 0.01$) in the hot plate test, and latency values in the tail immersion test ($P < 0.01$).

3.3. Effects of antisense oligodeoxynucleotides on BUBU-induced antinociception

Overall analysis showed significant antinociceptive effects in the tail immersion test in all the antisense oligodeoxynucleotide experiments. Control mice that received i.c.v. injections of vehicle, random-oligodeoxynucleotide or antisense oligodeoxynucleotides to opioid receptors showed a similar response in the tail immersion test with basal latencies comparable to those of non-injected (naive) mice. In agreement with the previous experiments, the administration of BUBU (1 μg, i.c.v.) produced a significant antinociceptive response in naive mice ($P < 0.05$). The antinociceptive effects produced by BUBU administration were similar in naive animals and in mice that received i.c.v. injections of vehicle or random-oligodeoxynucleotide, as previously reported when using a similar protocol with other antinociceptive compounds (Lai et al., 1994; Sánchez-Blazquez et al., 1995, 1997). Therefore, the responsiveness of mice in this nociceptive assay was not altered by the experimental procedure alone.

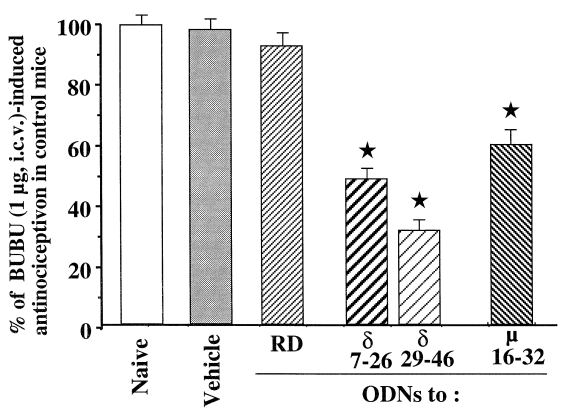

Fig. 3. Effect of sub-chronic i.c.v. administration of antisense oligodeoxynucleotides to μ- or δ-opioid receptor on the antinociception induced in the tail immersion test by central administration of BUBU (1 μg, i.c.v.). Mice were injected with vehicle, random-oligodeoxynucleotide (RD) or antisense oligodeoxynucleotides for 5 consecutive days, as reported in Section 2. Naive mice did not receive any treatment during these 5 days. Antinociception is expressed as the percentage of the antinociceptive response induced by BUBU in naive mice. Values are the mean ± S.E.M. from groups of 10–15 mice each. ★$P < 0.05$, vs. control groups receiving vehicle or random-oligodeoxynucleotide (Newman–Keul's test).

Mice subjected to sub-chronic administration of the antisense oligodeoxynucleotides showed an impaired antinociceptive response to the δ-opioid receptor agonist BUBU. Thus, BUBU antinociception was significantly ($P < 0.05$) reduced by antisense oligodeoxynucleotide to μ- 16–32 opioid receptor gene sequence. A strong decrease in BUBU-induced antinociception ($P < 0.05$) was also produced by antisense oligodeoxynucleotides to δ-7–26 or δ- 29–46 opioid receptor gene sequence (Fig. 3).

4. Discussion

The present results confirm the potent antinociceptive responses observed in rodents after BUBU administration (Baamonde et al., 1991; Delay-Goyet et al., 1991). These effects have now been obtained in both the tail immersion and the hot plate test parameters after systemic or central administration of BUBU. The possible involvement of μ-

and δ-opioid receptors in BUBU-induced antinociception under these experimental conditions has been first investigated using selective antagonists of μ-(cyprodime) and δ-(naltrindole) opioid receptors. For this purpose, cyprodime (Schmidhammer et al., 1989) and naltrindole (Portoghese et al., 1988) were selected since they show a high selectivity for μ- and δ-opioid receptors, respectively, and both are able to cross the blood–brain barrier. The doses of cyprodime (10 mg/kg, s.c.) and naltrindole (2.5 mg/kg, s.c.) used in this study have been previously shown to effectively block several pharmacological responses induced by the activation of μ- (Schmidhammer et al., 1989, 1990a) and δ-opioid receptors (Portoghese et al., 1988; Sofuoglu et al., 1991; Baamonde et al., 1992; Kalso et al., 1992; Lee et al., 1994; Smadja et al., 1995), respectively. BUBU (1 μg) was given by i.c.v. route in this experiment since it was the most effective route of administration in these conditions. Pre-treatment with the μ-opioid receptor selective antagonist cyprodime blocked the antinociceptive responses induced by central administration of BUBU in the hot plate and tail immersion tests, revealing that at least in part, BUBU mediates its effects through the activation of μ-opioid receptors. Pre-treatment with the δ-opioid receptor selective antagonist naltrindole did not significantly modify the antinociceptive effects induced by central BUBU administration in the hot plate test but attenuated its responses in the tail immersion test, indicating that the δ-opioid receptor is involved in the antinociceptive effects of BUBU on this nociceptive assay. Therefore, the findings obtained with these selective antagonists suggest that BUBU mediates its antinociceptive effects via activation of both μ- and δ-opioid receptors on the tail immersion test, whereas a predominant involvement of μ-opioid receptor was revealed in the hot plate test.

Further evidence for this dual receptor action of BUBU was clearly demonstrated by using antisense oligodeoxynucleotides that selectively blocked the expression of μ- and δ-opioid receptors. Considering the experimental complexity of the oligodeoxynucleotide procedure and taking into account the results of the previous antagonist study revealing a dual participation of μ- and δ-opioid receptors only in BUBU-induced antinociception in the tail immersion test, this nociceptive assay was choosen to evaluate the effects of antisense oligodeoxynucleotides on BUBU responses. The administration of antisense oligodeoxynucleotides to the μ-opioid receptor (16–32) significantly reduced the antinociceptive effects induced by central administration of BUBU in the tail immersion test. This antisense oligodeoxynucleotide has been previously reported to specifically reverse the antinociceptive responses induced by the μ-opioid selective agonist [D-Ala, ^{2}N-MePhe^{4}, Gly^{5}-ol]enkephalin (DAMGO) without producing any effect on deltorphin II and DPDPE-induced antinociception (Sánchez-Blazquez et al., 1997). A decrease in central BUBU administration-induced antinociceptive responses was also observed with each antisense oligodeoxynucleotide to the δ-opioid receptor (7–26 and 29–46). The efficacy and selectivity of identical antisense oligodeoxynucleotides to the δ-opioid receptor have been also tested in previous studies. Thus, antisense probes to nucleotides 29–46 of the murine δ-opioid receptor have been previously reported to selectively decrease antinociceptive responses induced by the selective δ-opioid receptor agonists DPDPE (a putative agonist of δ_1-opioid receptor) and deltorphin II (a putative agonist of δ_2-opioid receptor), whereas antisense probes to nucleotides 7–26 of the murine δ-opioid receptor only attenuated the antinociception induced by deltorphin II. These two antisense oligodeoxynucleotides to δ-opioid receptor had not effect on DAMGO-induced antinociception (Sánchez-Blazquez et al., 1997). Therefore, the profile of BUBU-induced antinociceptive responses defined by the administration of these antisense oligodeoxynucleotides was similar to deltorphin II. In agreement, BUBU has the conformational requirements to bind δ_2-opioid receptor (Hruby et al., 1994). Accordingly, with the profile of BUBU, the selective δ-opioid receptor antagonist used in this study, naltrindole, has also an apparent higher antagonistic action in vivo on δ_2-opioid receptor.

The use of a dual pharmacological (antagonists)/molecular (antisense oligodeoxynucleotides) procedure is important in this experimental approach due to the uncertainties of using pharmacological ligands, the selectivity of which on most in vivo pharmacological models remains to be clarified. This is particularly the case when using nociceptive assays that induce responses involving central nervous structures containing different proportions of opioid receptors distributed along the neuroaxis (Basbaum and Fields, 1984; Besson and Chaouch, 1987). The present study has demonstrated a remarkable similarity in the results obtained when using selective antagonists and antisense oligodeoxynucleotides, and clearly revealed the exact nature of BUBU receptor mediated antinociception. This dual strategy further validates the use of the antisense method in the investigation of central nervous system receptor mediated physiological and pharmacological responses.

Opioid compounds have been shown to produce their antinociceptive effects through action at both spinal (Ling and Pasternak, 1983) and supraspinal sites (Jensen and Yaksh, 1986). At the supraspinal level, μ-opioid receptor seems to be the most directly involved in the antinociceptive responses induced by exogenous (Chaillet et al., 1984, Baamonde et al., 1991) and endogenous opioids (Noble and Roques, 1995). At the spinal level, both μ- and δ-opioid receptors have been reported to play an important role in opioid-mediated antinociception (Dickenson, 1991; Dickenson et al., 1988; Sullivan et al., 1989). The relatively higher proportion of μ-opioid receptor densities in the brain and the elevated δ-opioid receptor concentration in the spinal cord provide another argument to reinforce

this view (Mansour and Watson, 1993). Taking into account this hypothesis, the differential involvement of spinal and supraspinal mechanisms in the two antinociceptive tests utilised in this study (Eddy and Leimbach, 1953; Janssen et al., 1963) may explain the differences seen between the effects of naltrindole after BUBU administration. Thus, the ability of naltrindole to block BUBU antinociception in the tail immersion but not in the hot plate test could be due to the participation of a spinal mechanism in the nociceptive responses produced by the tail immersion test, whereas the responses in the hot plate test require a supraspinal integration. However, the results obtained in mice receiving a central administration of antisense oligodeoxynucleotides to δ-opioid receptor also suggest an involvement of supraspinal δ-opioid receptors on BUBU-induced antinociception (Sánchez-Blazquez et al., 1997). In agreement, previous pharmacological evidence have indicated that activation of the δ-opioid receptor could participate in the thermal antinociceptive responses of the selective δ-opioid receptor agonists DPDPE, Tyr-D-Ala-Gly-Phe-D-Leu (DADLE) and Tyr-D-Thr-Gly-Phe-Leu-Thr (DTLET), at the supraspinal level (Porreca et al., 1984, 1987; Heyman et al., 1987; Sánchez-Blazquez and Garzón, 1989). However, some controversial results have been previously reported on the reversal of δ-opioid receptor agonists-induced antinociception by selective antagonists for this receptor, which could be probably related to experimental variables such as the species, strength of the thermal stimuli and the concentrations of pharmacological agonists and antagonists used.

The apparent dichotomy in selectivity of ligands at opioid receptors on in vitro bioassay analysis and in vivo studies is now an important consideration when using these compounds. Thus, many previous investigations have indicated that several highly selective peptide δ-opioid receptor agonists mediate some of their antinociceptive effects through the activation of the μ-opioid receptor (Cowan et al., 1985; Shook et al., 1987; Shah et al., 1994). Accordingly, although BUBU clearly shows higher relative selectivity for δ- than for μ-opioid receptors using in vitro bioassay analysis (Gacel et al., 1988) and binding studies (Delay-Goyet et al., 1991), the present results have implicated a functional activity of BUBU in vivo at both the μ- and δ-opioid receptors. This finding suggests that the intrinsic efficacy of BUBU in vivo at μ- and δ-opioid receptors could be different from the in vitro binding selectivity at each site.

In conclusion, the present findings using selective μ- and δ-opioid receptor antagonists as well as antisense oligodeoxynucleotides blocking μ- and δ-opioid receptor expression, indicate that BUBU mediates its antinociceptive effects via activation of both μ- and δ-opioid receptors. This dual strategy resulted in a similar result when using both experimental approches which has clearly allowed the definition of the exact involvement of each opioid receptor in this pharmacological response of BUBU. The dual strategy used in this study can be also applied to evaluate the mechanisms involved in the responses of other novel analgesic compounds.

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