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## Pharmacological characterization of [D-Ala<sup>2</sup>,Leu<sup>5</sup>,Ser<sup>6</sup>]enkephalin (DALES): antinociceptive actions at the $\delta_{\text{non-complexed}}$ -opioid receptor

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Substantial evidence has been accumulated which suggests that opioid  $\delta$  receptors may be distinguished on the basis of their involvement in the modulation (i.e., increase or decrease in potency) of  $\mu$ -mediated antinociception. On this basis, it has been hypothesized that some opioid  $\delta$  receptors exist within a functional complex with  $\mu$  receptors ( $\delta_{\text{complexed}}$  ( $\delta_{\text{cx}}$ ) receptors) while other  $\delta$  sites do not ( $\delta_{\text{non-complexed}}$  ( $\delta_{\text{ncx}}$ ) receptors). Recent work with [D-Ala<sup>2</sup>,Leu<sup>5</sup>,Cys<sup>6</sup>]enkephalin (DALCE) has demonstrated that this compound produces initial antinociceptive actions, does not modulate morphine antinociception and appears to bind irreversibly to the  $\delta_{\text{ncx}}$  site, presumably by means of thiol-disulfide exchange between the receptor and the cysteine sulfhydryl group. To determine if a structural basis exists for actions at the hypothesized  $\delta_{\text{ncx}}$  receptor, in the present study we report the synthesis and pharmacological characterization of [D-Ala<sup>2</sup>,Leu<sup>5</sup>,Ser<sup>6</sup>]enkephalin (DALES), a close structural analogue of DALCE. If a structural basis for action at the  $\delta_{\text{ncx}}$  site exists, then DALES would be predicted to produce antinociception, fail to modulate morphine antinociception and, since it lacks the free sulfhydryl group present in DALCE, fail to exhibit irreversible antagonistic actions; these predictions were supported. Additionally, pretreatment with DALCE at  $-24$  h, but not with DALES, blocked DALES-induced antinociception. These observations *in vivo* support the concept of a structural basis for activity at the hypothesized  $\delta_{\text{ncx}}$  site and suggest that DALES, like DALCE, may be a useful probe for pharmacological characterization of putative  $\delta$  receptor subtypes.

Opioid antinociception;  $\delta$ -Opioid receptors;  $\mu$ -Opioid receptors; Enkephalins;  
DALES ([D-Ala<sup>2</sup>,Leu<sup>5</sup>,Ser<sup>6</sup>]enkephalin); (Intracerebroventricular)

### 1. Introduction

The multiplicity of opioid receptors has been well accepted since the initial suggestion of multiple opioid receptors by Martin et al. (1976). While uncertainty still exists as to the specific physiological functions of opioid receptors, pharmacological evidence has accumulated which strongly implicate  $\delta$ , as well as  $\mu$ , receptors in supraspinal antinociception in the mouse (Heyman et al., 1987; Heyman et al., 1988). Additionally, data have also been accumulated which indicate that in some cases, opioid  $\mu$  and  $\delta$  receptors may be involved in functional interactions *in vitro* (e.g., Rothman and Westfall, 1982) and *in vivo* (Heyman et al., 1989a,b; Jiang et al., 1990a,b; and see Holaday et al., 1985 for review).

Early observations demonstrated that the proposed endogenous ligands for the opioid  $\delta$  receptor, [Leu<sup>5</sup>]en-

kephalin and [Met<sup>5</sup>]enkephalin, respectively produced an increase and decrease in morphine antinociceptive potency (Vaught and Takemori, 1979; Lee et al., 1980). These observations have been confirmed with other synthetic  $\delta$  ligands *in vivo* (Heyman et al., 1989a,b) as well as with radioligand binding techniques *in vitro* (Rothman et al., 1988) and have supported the suggestion that endogenous opioids may function in a modulatory capacity for  $\mu$ -mediated analgesia (Barrett and Vaught, 1982). The hypothesis of functional or physical interactions between  $\mu$  and  $\delta$  opioid receptors has been extended to suggest that some opioid  $\delta$  receptors may exist within a  $\mu$ - $\delta$  complex (i.e.,  $\delta_{\text{complexed}}$  ( $\delta_{\text{cx}}$ ) receptors) while other  $\delta$  receptors do not (i.e.,  $\delta_{\text{non-complexed}}$  ( $\delta_{\text{ncx}}$ ) receptors) (Rothman et al., 1988).

Substantial data *in vivo* are consistent with such a hypothesis. In this regard, doses of  $\delta$  agonists such as [D-Pen<sup>2</sup>,D-Pen<sup>5</sup>]enkephalin (DPDPE) or [D-Ala<sup>2</sup>,Met<sup>5</sup>]enkephalinamide (DAMA) which do not produce antinociceptive actions alone, respectively increase and decrease the antinociceptive potency of intracerebroventricular (i.c.v.) morphine in mice (Heyman et al.,

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1989a,b). Such positive (i.e., increase in morphine potency) or negative (i.e., decrease in morphine potency) modulation of morphine antinociception can be blocked by ICI 174,864, a highly selective  $\delta$  antagonist (Cotton et al., 1984), which itself neither antagonizes nor modulates morphine antinociception. In addition to the modulation of potency by  $\delta$  agonists, recent work has also demonstrated a modulation of the efficacy of  $\mu$ -induced antinociception (Jiang et al., 1990b).

Support for this hypothesis has also been obtained from data with [D-Ala<sup>2</sup>,Leu<sup>5</sup>,Cys<sup>6</sup>]enkephalin (DALCE) (Bowen et al., 1987), a novel peptide, which produces initial reversible antinociception which can be antagonized by the  $\delta$ -antagonist, ICI 174,864 followed by a long-lasting (irreversible) antagonism at  $\delta$  sites (Calcagnetti et al., 1989; Jiang et al., 1990b). However, unlike DPDPE, DALCE did not modulate morphine antinociception, or block the modulation of morphine antinociception produced by DPDPE or by [Met<sup>5</sup>]enkephalin, suggesting that this compound acts selectively as a reversible agonist and irreversible antagonist at the hypothesized  $\delta_{ncx}$  receptor (Jiang et al., 1990b). Such a profile for DALCE in vivo is consistent with the observations of covalent binding to opioid  $\delta$  receptors in rat brain membranes, presumably by thiol-disulfide exchange between the receptor and the sulfhydryl group in the cysteine residue (Bowen et al., 1987).

Identification of structural characteristics producing selectivity for the proposed  $\delta$  receptor subtypes would greatly strengthen the hypothesis. Therefore, we report the synthesis and pharmacological characterization of [D-Ala<sup>2</sup>,Leu<sup>5</sup>,Ser<sup>6</sup>]enkephalin (DALES), a close structural analogue of DALCE in which serine is substituted for cysteine in position 6. Such a modification should retain selectivity for the  $\delta_{ncx}$  site, but prevent the irreversible actions associated with DALCE. Additionally, DALES should produce direct antinociceptive actions which could be blocked by pretreatment with DALCE. Finally, if DALES exerts selectivity for the  $\delta_{ncx}$  site, it should not produce modulatory actions on morphine antinociception. The present study provides evidence which supports these predictions suggesting that a structural basis for selective actions at the putative  $\delta_{ncx}$  receptor may exist.

## 2. Materials and methods

### 2.1. Animals

Male, ICR mice weighing 20-30 g were used in these studies. They were housed in groups of five in plexiglass cages and maintained in light and temperature con-

trolled rooms. They were given laboratory chow and tap water ad libitum.

### 2.2. Intracerebroventricular (i.c.v.) injections

I.c.v. injections were given directly into the left lateral ventricle according to methods previously described (Haley and McCormick, 1957; Porreca et al., 1984). Under light ether anesthesia, a small incision was made in the scalp. Using a 10  $\mu$ l Hamilton syringe with a 26 gauge needle, an injection volume of 5  $\mu$ l was delivered 2 mm lateral and 2 mm caudal to bregma at a depth of 3 mm.

### 2.3. Antinociceptive assay

Antinociception was assessed by recording the latency to a rapid tail flick using 55°C warm water as the nociceptive stimulus (Heyman et al., 1986). Prior to drug administration, control latencies were determined and mice not responding within 5 s were eliminated from the study. The maximal latency allowed was 15 s (cut-off time). Scores for antinociception were calculated according to the following formula: % antinociception = 100  $\times$  (test latency - control latency)/(15 - control latency).

Antinociception for i.c.v. DALES was determined at 5, 10, 20, 40 and 60 min. From the time course, +10 min was chosen as the time of peak effect, similar to that for the antinociceptive actions of morphine (Heyman et al., 1986; 1989a,b; Jiang et al., 1990a,b). Consequently, in subsequent experiments antinociception was evaluated at +10 min. Pretreatments with i.c.v. DALES and DALCE were made 24 h prior to testing, a time previously shown to produce maximal antagonist action for DALCE (Jiang et al., 1990a).

### 2.4. Chemicals

Morphine sulfate (Mallinckrodt Chemical Co., St. Louis, MO) and ICI 174,864 (N,N-diallyl-Tyr-Aib-Aib-Phe-Leu-OH, where Aib is  $\alpha$ -aminoisobutyric acid) (Cambridge Research Biochemicals, Atlantic Beach, NY) were purchased. DPDPE, DALCE, and DALES were synthesized using previously described methods (Mosberg et al., 1983; Bowen et al., 1987).

### 2.5. Statistics

Data are represented as the mean  $\pm$  S.E.M. for groups of 10 mice. Regression lines, A<sub>50</sub>s and their 95% confidence limits (C.L.) were determined using the computer program of Tallarida and Murray (1986) (procedure 8).

### 3. Results

#### 3.1. Dose- and time-related antinociceptive effects of DALES

In response to warm water thermal stimuli, i.c.v. DALES produced dose and time-related antinociceptive effects (fig. 1). Peak effects occurred at +10 min and antinociception generally subsided by 40 min. Antinociceptive scores obtained at 10 min for 1.52 nmol and 4.57 nmol DALES (95.8 and 100%, respectively) were not significantly different; however, the higher dose produced a longer lasting effect. When compared to the acute antinociceptive effects of DALCE at +10 min, the dose-response lines for these compounds were parallel and indicated that DALCE and DALES were nearly equipotent; the  $A_{50}$  value (and 95% C.L.) for DALES was 0.26 (0.17-0.38) which compares favorably to the  $A_{50}$  value of DALCE of 0.32 (0.18-0.56) nmol reported by Jiang et al. (1990a).

#### 3.2. Effects of $\delta$ antagonists on DALES antinociception

Co-administration of ICI 174,864 (4.4 nmol) antagonized the antinociceptive effects of DALES, in agreement with the ability of this  $\delta$  antagonist to antagonize the acute antinociception associated with DALCE (Jiang et al., 1990a) and indicating that this effect is mediated through  $\delta$  receptors (fig. 2). Pretreatment of mice with i.c.v. DALCE (4.57 nmol at -24 h) also blocked DALES antinociception (fig. 3), in agreement with the observation that DALCE pretreatment blocks the acute antinociceptive actions of DALCE itself, as well as those of DPDPE (Jiang et al., 1990a). In contrast, pretreatment with DALES (4.57 nmol at -24 h) did not block DALES induced antinociception (data not shown).

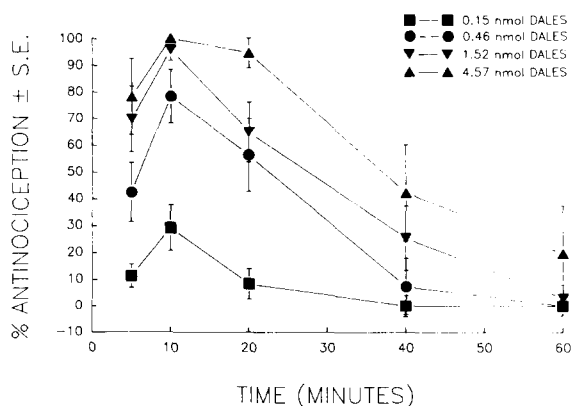


Fig. 1. Dose- and time-related antinociceptive effects of i.c.v. DALES in the 55°C warm-water tail flick assay (0.15 nmol, squares; 0.46 nmol, circles; 1.52 nmol, inverted triangles; 4.57 nmol, triangles).

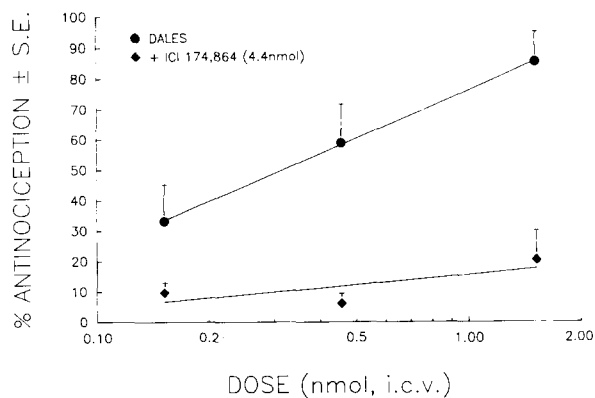


Fig. 2. I.c.v. antinociceptive dose response lines at +10 min in the 55°C warm-water tail flick assay for DALES alone (circles) and after co-administration with ICI 174,864 (diamonds).

#### 3.3. Lack of modulation of morphine antinociception by DALES

Administration of DALES (0.06 nmol) at a dose which was sub-effective in producing antinociception (i.e., less than 10% antinociception, extrapolated from the dose-response line), failed to produce either positive or negative modulation of morphine antinociception (fig. 4).

### 4. Discussion

Opioid  $\mu$  agonists such as morphine have been shown to produce effects in mice that can be modulated by  $\delta$  agonists (Vaught and Takemori, 1979; Lee et al., 1980; Heyman et al., 1989a,b; Jiang et al., 1990a,b). Such data have led to the hypothesis of  $\delta$  receptors existing in a functional or physical complex with  $\mu$  receptors (Rothman et al., 1988; Schoffelmeer et al., 1987; 1990). According to such a hypothesis, agonists which interact

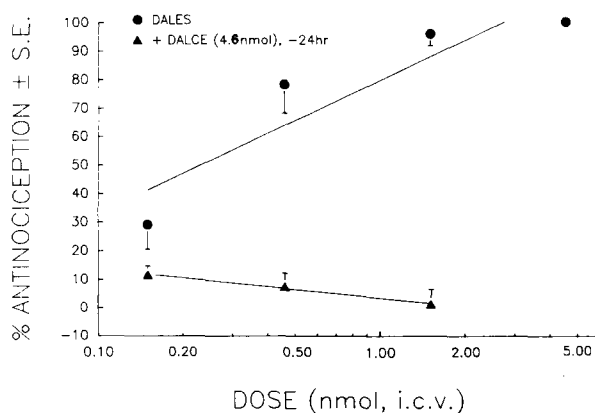


Fig. 3. Antinociceptive dose-response lines for i.c.v. DALES alone (circles) and after i.c.v. pretreatment with DALCE (4.57 nmol at -24 h) (triangles).

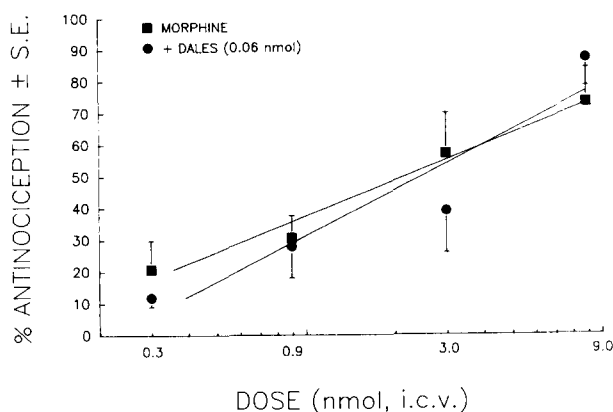


Fig. 4. Antinociceptive dose response lines for i.c.v. morphine alone (squares) and in the presence of sub-antinociceptive dose of i.c.v. DALES of 0.06 nmol (circles).

with  $\delta_{cx}$  receptors should modulate morphine antinociception, in either a positive or negative fashion. Conversely,  $\delta$  agonists which act at  $\delta_{ncx}$  receptors should not modulate morphine-induced antinociception, although such agonists would be expected to produce direct antinociception through  $\delta_{ncx}$  receptors. Compounds such as DPDPE produce both direct antinociceptive actions and modulation of morphine antinociception, and may thus be non-selective for these sites. In contrast, our recent findings with DALCE, suggest that this compound is selective for the hypothesized  $\delta_{ncx}$  receptors. In an attempt to further test the hypothesis of subtypes of  $\delta$  receptors which can be distinguished on the basis of possible interaction with  $\mu$  agonists, we have synthesized DALES, a close structural analogue of DALCE.

We predicted that if a structural basis existed for ligands which act at the  $\delta_{ncx}$  receptor, then DALES should retain selectivity at this site as it differs from DALCE only in the substitution of a serine for a cysteine residue at the C terminus. Like DALCE (Jiang et al., 1990a), DALES produced dose- and time-related antinociception that was antagonized by the selective  $\delta$  antagonist, ICI 174,864. As expected from their structural similarities, the antinociceptive actions of DALES were blocked in mice pretreated at  $-24$  h with DALCE. This observation was in agreement with previous experiments which demonstrated that DALCE pretreatment blocked the antinociceptive actions of DALCE itself (Jiang et al., 1990a), and indicates that these ligands bind to the same receptor. Also as predicted, substitution of serine for cysteine at the C terminus, prevented DALES from exhibiting the irreversible profile associated with DALCE. Therefore, as expected, DALES pretreatment at  $-24$  h did not block antinociception produced by subsequent DALES administration. The lack of modulation of morphine antinociception by DALES in this report also indicates that DALES, like

DALCE, does not interact the  $\delta_{cx}$  receptor. This finding provides further support for the concept of  $\delta$  receptor subtypes.

The involvement of multiple opioid receptors in antinociception (see Knapp et al., 1989, for review) offers the possibility that future clinical management of pain may be enhanced by the development of more selective opioids with improved efficacy and decreased risk of tolerance and/or dependence. Towards this goal,  $\delta$  agonists offer significant promise as they produce antinociception directly at spinal and supraspinal levels and can modulate both the potency and efficacy of  $\mu$  agonists (Heyman et al., 1988; Jiang et al., 1990a). Consequently, the development of pharmacological tools such as DALES and DALCE can reveal insights into the potential significance of activity at putative subtypes of  $\delta$  receptors.

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