Antinociceptive and Respiratory Effects of Intrathecal H-Tyr-D-Arg-Phe-Lys-NH₂ (DALDA) and [Dmt¹]DALDA

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ABSTRACT

DALDA (H-Tyr-D-Arg-Phe-Lys-NH₂) and [Dmt¹]DALDA (H-Dmt-D-Arg-Phe-Lys-NH₂) are potent and highly selective μ-opioid agonists (Kᵢ/Kᵢ > 10,000 and Kᵢ/Kᵢ > 100). Both peptides carry a 3+ charge at physiological pH. Their antinociceptive and respiratory effects were compared with morphine (MOR) after intrathecal administration in rats. Both DALDA and [Dmt¹]DALDA produced dose-dependent and naloxone-reversible antinociceptive effects with relative potencies of 14 and 3000 that of MOR. The antinociceptive potency of [Dmt¹]DALDA far exceeded its affinity and potency at the μ-opioid receptor and may be explained by its ability to inhibit norepinephrine (NE) uptake in spinal cord synaptosomes. The antinociceptive response to [Dmt¹]DALDA was significantly attenuated by the α₂-adrenergic antagonist yohimbine. Thus, [Dmt¹]DALDA may be regarded as a drug with dual actions, and its antinociceptive potency is better described by both its affinity and potency at μ-opioid receptors, and its potency at inhibiting NE uptake. The analgesic duration of an equipotent dose of MOR, DALDA, and [Dmt¹]DALDA was 3, 7, and 13 h, respectively, and the long duration may be due to the hydrophilic nature of these peptide analogs. Respiratory effects were determined using whole body plethysmography at 3 and 30 x the antinociceptive ED₅₀. A significant depression in minute ventilation was observed with the higher dose of morphine and both doses of DALDA, but not with either dose of [Dmt¹]DALDA. Because of its high antinociceptive potency, long duration of action, and low propensity to induce respiratory depression, [Dmt¹]DALDA is of interest as a drug candidate for intrathecal analgesia.

The intrathecal administration of opioids is a useful technique in pain management. It is used in the management of postoperative pain, cancer pain, and pain associated with labor and delivery. Presently, the most often-used intrathecal opioids include morphine, fentanyl, and sufentanil. Following distribution within the cerebrospinal fluid (CSF), the opioids penetrate through the pia mater and into the spinal cord where they act on opioid receptors in the superficial laminae of the dorsal horn. Lipid solubility is the major determinant of the rate of onset of opioid action: the more lipid soluble the opioid, the more rapid the onset of action. Thus, sufentanil has a much more rapid onset of action than fentanyl or morphine. However, the physicochemical properties of opioids do not only determine their rate of absorption but also their clearance from CSF into the vascular system and their distribution within the CSF. The increased lipid solubility results in increased systemic absorption, which limits the duration of analgesia. Although morphine has a longer duration of action than fentanyl, it is still too short-acting for many purposes and repeat dosing or intrathecal infusion is often required. In addition, the slow clearance of hydrophilic drugs from CSF allows for rostral spread of the opioid to medullary respiratory centers, thereby resulting in respiratory depression. Although respiratory depression is less of a problem with the more lipophilic drugs, delayed respiratory depression is the most dangerous side effect after intrathecal morphine (Cousins and Mather, 1984).

DALDA (H-Tyr-D-Arg-Phe-Lys-NH₂) and [Dmt¹]DALDA (H-Dmt-D-Arg-Phe-Lys-NH₂) are derrmorphin analogs with extraordinary selectivity for the μ-opioid receptor (Kᵢ/Kᵢ > 10,000 and Kᵢ/Kᵢ > 100) (Table 1) (Schiller et al., 1989, 1999, 2000). There are some differences between the two peptide analogs, with...
[Dmt\(^1\)DALDA] having 10-fold higher affinity for the \(\mu\)-opioid receptor and 200-fold higher potency in the guinea pig ileum (GPI) assay. Both DALDA and [Dmt\(^1\)DALDA] show higher potency in the mouse vas deferens (MVD) assay than would be expected on the basis of their very low \(\delta\)-receptor binding affinities. This is due to the fact that although the MVD contains predominantly \(\delta\)-opioid receptors, it also has \(\mu\)-opioid receptors. [Dmt\(^1\)DALDA] is even 20 times more potent than morphine and 5 times more potent than endomorphin II in the GPI assay. DALDA and [Dmt\(^1\)DALDA] have different physicochemical properties compared with other opioid peptide analogs in that they carry a net positive charge (3+) at physiological pH and are thus hydrophilic and more polar than morphine. The polar characteristic of these peptide analogs has been confirmed by their capacity factors (\(k'\)) as determined by high performance liquid chromatography (Schiller et al., 2000). Thus, it may be anticipated that there will be a difference in their distribution and clearance after intrathecal administration compared with morphine. This should affect their efficacy in producing analgesic effects and their likelihood to produce supraspinal side effects such as delayed respiratory depression.

The present study was designed to characterize the antinociceptive effects of DALDA and [Dmt\(^1\)DALDA] after intrathecal administration. The potency and time course of the antinociceptive actions of intrathecal DALDA and [Dmt\(^1\)DALDA] were determined in the rat tail-flick test and compared with those of morphine. Furthermore, we investigated the respiratory effects of intrathecal DALDA and [Dmt\(^1\)DALDA] in comparison with morphine to determine whether these opioid peptides are more likely to produce delayed respiratory depression. Unexpectedly, our data showed that DALDA and [Dmt\(^1\)DALDA] were 14- and 3000-fold more potent than morphine, respectively. The extraordinary potency of [Dmt\(^1\)DALDA] cannot be explained by its affinity or potency at the \(\mu\)-opioid receptor. This suggested to us that [Dmt\(^1\)DALDA] might have other nonopioid actions that potentiate its opioid antinociceptive action. Spinal opioid analgesia is known to be modulated by the \(\alpha_2\)-adrenergic receptor and serotonin receptor. Concurrent administration of norepinephrine (NE) or serotonin (5-HT) uptake inhibitors, or adrenergic and serotonergic agonists, can result in a synergistic interaction with \(\mu\)-opioid agonists (Larson and Takemori, 1977; Yaksh and Reddy, 1981; Loomis et al., 1987; Wilcox et al., 1987; Reimann et al., 1999). We now have evidence that DALDA and [Dmt\(^1\)DALDA] can inhibit NE uptake in spinal cord synaptosomes, with [Dmt\(^1\)DALDA] being 13-fold more potent than DALDA and 212-fold more potent than morphine. Our data suggest that enhancement of endogenous extraneuronal NE levels may possibly account for the extraordinary potency of [Dmt\(^1\)DALDA] when administered intrathecally.

### Materials and Methods

#### Animals

Male Sprague-Dawley rats (300–350 g) were used. All experiments were conducted in accordance with the ethical guidelines of International Association for the Study of Pain and were approved by the Institutional Animal Use Committee of Chiba University School of Medicine.

#### Drugs and Chemicals

Morphine hydrochloride (MOR) was obtained from Takeda, Osaka, Japan. DALDA and [Dmt\(^1\)DALDA] were synthesized by methods described elsewhere (Schiller et al., 1989, 2000). Naloxone hydrochloride was obtained from Sigma, St. Louis, MO. Each drug was dissolved in saline. 1-[7,8-\(^3\)H]Norepinephrine (specific activity 1.37 TBq/mmol, radiochemical purity 93.2%), and 5-hydroxy[\(^1\)H]tryptamine trifluoroacetic acid (specific activity 4.00 TBq/mmol, radiochemical purity 99%) were purchased from Amersham Pharmacia Biotech (Buckinghamshire, England).

#### Drug Administration

Intrathecal delivery was performed either via an indwelling intrathecal catheter or by direct injection depending on the study. The indwelling catheter is preferable for repetitive drug administration, whereas direct percutaneous injection was used for the respiratory studies because of the possibility that the intrathecal catheter may impede rostral distribution of the drugs.

#### Via Intrathecal Catheter

Intrathecal catheterization was performed at least 2 days before the experiment as previously described (Shimoyama et al., 1996). Briefly, under halothane anesthesia, a PE-10 tube was inserted through a small hole made in the atlanto-occipital membrane and threaded 8.5 cm down the intrathecal space to the lumbosacral level of the spinal cord. Drugs were delivered via the catheter in a volume of 5 \(\mu\)l (except for study 5), followed by 10 \(\mu\)l of saline to flush the catheter.

#### Direct Percutaneous Injection

Under light halothane anesthesia a needle connected to a Hamilton syringe was inserted percutaneously between spinal processes of the 3rd and 4th lumbar vertebrae into the intrathecal space. A quick flick of the tail was observed when the tip of the needle entered the intrathecal space and was used as an indicator of successful puncture (Mestre et al., 1994). Drugs were delivered in a volume of 5 \(\mu\)l.

#### Analgesic Testing

To assess the antinociceptive effects of the opioids, the tail-flick test was used. Radiant heat was applied to the tail at 5 to 8 cm from the tip using a tail-flick apparatus (ITC, Woodland Hills, CA). The time from the onset of the heat to the withdrawal of the tail (tail-flick...
latency) was measured. The intensity of the radiant heat was adjusted so that baseline latencies would fall between 2.5 and 3.5 s. To avoid tissue damage the heat stimulus was discontinued after 7 s. A baseline latency was obtained for each animal before the administration of any drug. Subsequent response latencies were determined at designated time points. Analgesic testing was performed by a blinded investigator.

Study 1: Analgesic Potencies of Intrathecal MOR, DALDA, and [Dmt₁]DALDA. Cumulative dose-response studies were performed for each drug using the tail-flick test (Shimoyama et al., 1996, 1997a). Each drug was tested in a group of 10 rats. After measuring the baseline latencies, increasing doses of each drug were administered via an intrathecal catheter until each animal in the group became an analgesic responder. An analgesic responder was defined as one whose response tail-flick latency was 2 or more times the value of the baseline latency. The response latency after each dosing was determined at peak analgesia, which was 15, 30, and 45 min after the administration of the MOR, DALDA, and [Dmt₁]DALDA, respectively (based on preliminary studies). Any subsequent dosing was performed immediately after the determination of response latency. The percentage of analgesic responders in the group of rats for each cumulative dose was calculated, and a cumulative dose-response curve was constructed. The dose-response data were analyzed by the BLISS-21 computer program. This program maximized the log-likelihood function to fit a parallel set of Gaussian normal sigmoid curves to the dose-response data and provides ED₅₀ values, 95% confidence interval, and relative potency estimates (Umans and Inturrisi, 1981).

Study 2: Naloxone Reversal of Antinociceptive Effects. The effect of naloxone on the antinociceptive effects of intrathecal MOR, DALDA, and [Dmt₁]DALDA was examined. An equipotent dose of MOR (10 nmol), DALDA (0.7 nmol), or [Dmt₁]DALDA (3.4 pmol) was given via an intrathecal catheter. Tail-flick latencies were measured before the administration of any drug and at the time of peak analgesia for each compound (at 15, 30, and 45 min after administration, for MOR, DALDA, and [Dmt₁]DALDA, respectively). Naloxone hydrochloride at a dose of 82.5 nmol (Tiseo and Yaksh, 1993) or saline was administered via the intrathecal catheter 10 min before the second tail-flick testing. Four rats were tested for each combination of drugs. Data were analyzed using the paired t test.

Study 3: Time Course of Antinociceptive Effects. Equipotent doses (3 times the ED₅₀ value obtained in study 1) of MOR, DALDA, and [Dmt₁]DALDA were given intrathecally by direct percutaneous injection. Tail-flick latencies were measured before and every hour up to 15 h after the administration of each drug. The number of rats tested for each drug was eight. Data were analyzed using the one-way analysis of variance followed by the Dunnett’s test.

Study 4: Respiratory Effects of Intrathecal MOR, DALDA, and [Dmt₁]DALDA. The effects of each drug on minute ventilation (VE) under 5% CO₂ challenge were evaluated using whole body plethysmography (Tatsumi et al., 1991). An unrestrained rat was placed in a 3-liter whole body plethysmograph chamber. After a 15-min acclimation period, a gas mixture of 5% CO₂ and 21% O₂ in N₂ (100% humidified) was supplied into and out of the chamber at a rate of 1000 ml/min, and the animal was allowed to breathe the gas mixture for 5 min. After steady-state ventilatory condition had been reached and with the animal awake and quiet, the inlet and outlet of the chamber were closed and pressure changes in the box (due to the warming and wetting of the gas inspired by the rat and the cooling and drying of the expired gas) were recorded using a high-gain differential pressure transducer. A calibration volume of 0.2 ml of air was regularly introduced into the chamber during the recordings. The recordings were made for 20 to 30 s. Tidal volumes were calculated from the pressure changes using the equation derived by Drorbaugh and Fenn (1955). Respiratory frequencies were determined from the number of respiratory cycles in the recordings and VE values were calculated (tidal volume × frequency).

Rats were randomly assigned to one of seven groups. The number of rats in each group was 6 to 10. The animals of each group were given 3 or 30 times the ED₅₀ value (antinociceptive) of MOR, DALDA, or [Dmt₁]DALDA, or saline by direct percutaneous intrathecal injection. VE under 5% CO₂ challenge was determined before and every hour up to 10 h after the administration of the drug. VE values were expressed as a percentage of the baseline VE value obtained before the administration of any drug. The smallest VE value obtained after the administration of a drug (minimum VE) was determined for each animal. Mean values of the minimum Vₑ for animals of each group were compared using the one-way analysis of variance followed by the Dunnett’s test. For each animal, respiratory depression was defined as a minimum Vₑ that is below two standard deviations of the mean (mean – 2 × S.D.) of the saline group.

Study 5: Effect of α₂-Adrenergic Blockade on Antinociceptive Action of [Dmt₁]DALDA and DALDA. The antinociceptive effect of intrathecal [Dmt₁]DALDA and DALDA were compared in the absence and presence of intrathecal yohimbine, an α₂-adrenergic antagonist. Rats were administered [Dmt₁]DALDA alone (1.1 pmol, n = 10), [Dmt₁]DALDA (1.1 pmol) and yohimbine (100 μg) (n = 10), DALDA alone (240 pmol, n = 9), DALDA (240 pmol) and yohimbine (100 μg) (n = 9), or yohimbine alone (100 μg) (n = 6). Due to the limited solubility of yohimbine, the drug solutions were prepared with 50% dimethyl sulfoxide in saline and delivered in a volume of 10 μl. Intrathecal administration of 10 μl of 50% dimethyl sulfoxide in saline by itself did not have any effect on tail-flick latency (n = 4, data not shown). Tail-flick latencies were measured before and every 20 min up to 120 min after drug administration. Within-group differences were analyzed by one-way ANOVA and between-group differences were analyzed by two-way ANOVA.

Inhibition of NE and 5-HT Uptake in Spinal Cord Synaptosomes

A crude synaptosomal (P2) fraction was prepared from spinal cords as described previously (Lonart and Johnson, 1995; Li et al., 2000). Briefly, the tissue was minced and homogenized in 10 ml of ice-cold 0.32 M sucrose solution (pH 7.4) with a Thomas B075 homogenizer, clearance 0.13 to 0.18 mm. The homogenate was centrifuged for 10 min at 1200g at 4°C. The resulting supernatant was then centrifuged at 20,000g for 20 min at 4°C and the supernatant discarded. The pellet (P2) was gently resuspended in aerated (100% O₂) ice-cold buffer containing 20 mM HEPES, 140 mM NaCl, 5 mM KCl, 5 mM NaHCO₃, 1 mM MgCl₂, 1.2 mM Na₂HPO₄, 1.2 mM CaCl₂, and 10 mM glucose, pH 7.4, and centrifuged at 3000g for 12 min at 4°C. Synaptosomes were resuspended in 10 ml of buffer just before use. Protein concentration was determined by the method of Bradford (1976).

Synaptosomes were preincubated with varying concentrations of morphine, DALDA, or [Dmt₁]DALDA for 10 min at 37°C. [³H]NE (100 nM) or [³H]5-HT (50 nM) was then added and the incubation continued for 6 min before being terminated by rapid cooling in ice-cold water for 3 min. The synaptosomes were then collected using a Brandel Cell Harvester with GF/B filter and washed three times with ice-cold 150 mM Tris HCl buffer (pH 7.4). Filter-bound radioactivity was counted and the difference in [³H]NE accumulation at 37°C and 0°C was taken as a measure of active uptake. The results are presented as mean ± S.E.M. of three to five experiments, each carried out in triplicate.

Results

Study 1: Analgesic Potencies. MOR, DALDA, and [Dmt₁]DALDA each produced a dose-dependent antinociceptive effect in the tail-flick test (Fig. 1). ED₅₀ values and potency ratios obtained from the quantal dose-response curves are shown in Table 2. DALDA was 14 times more...
potent than MOR, whereas [Dmt1]DALDA showed a 3000-fold greater potency compared with MOR.

**Study 2: Naloxone Reversal of Antinociceptive Effects.** In rats that received MOR, DALDA, or [Dmt1]DALDA followed by saline, all tail-flick latencies reached cut-off (7 s) at the time of peak effect of each agonist. When naloxone (82.5 nmol) was administered instead of saline, the tail-flick latencies measured at the time of peak effect were not different from baseline values (Table 3).

**Study 3: Time Course of Antinociceptive Effects.** As shown in Fig. 2, MOR, DALDA, and [Dmt1]DALDA showed different time courses of antinociceptive effects after intrathecal administration of equipotent doses of the three drugs (3 times the ED_{50} value for antinociceptive effect). The tail-flick latencies were significantly greater than baseline for 3, 7, and 13 h after the intrathecal administration of MOR, DALDA, and [Dmt1]DALDA, respectively. All tail-flick latencies returned to baseline by the end of the experiment.

**Study 4: Respiratory Effects.** The effects of MOR, DALDA, and [Dmt1]DALDA on minimum V˙E are illustrated in Fig. 3. Compared with the group that received saline, the minimum VE was significantly lower in the group that received the high dose of MOR (30 times the antinociceptive ED_{50}) but not in the group that received the low dose of MOR (3 times the antinociceptive ED_{50}). Both the low- and high-dose DALDA groups showed a significantly lower minimum VE. In contrast, the minimum VE was not different in the low- or high-dose of [Dmt1]DALDA. When the minimum VE of each animal was checked to determine whether it satisfied the criterion set for respiratory depression [less than a critical value of mean − (2 × S.D.) of the minimum VE of the saline group], a substantial number of animals in the groups that showed a decrease in the mean value of minimum VE satisfied the criterion set for respiratory depression.

**TABLE 2**

<table>
<thead>
<tr>
<th>Compound</th>
<th>ED_{50} (pmol)</th>
<th>95% CI</th>
<th>Potency Ratios</th>
</tr>
</thead>
<tbody>
<tr>
<td>MOR</td>
<td>3330</td>
<td>1940–5600</td>
<td>1</td>
</tr>
<tr>
<td>DALDA</td>
<td>237</td>
<td>149–370</td>
<td>14.1</td>
</tr>
<tr>
<td>[Dmt1]DALDA</td>
<td>1.06</td>
<td>0.64–1.71</td>
<td>3160</td>
</tr>
</tbody>
</table>

**TABLE 3**

Reversal of antinociceptive effects of MOR, DALDA, and [Dmt1]DALDA by naloxone

A dose 3 times the ED_{50} value of MOR, DALDA, or [Dmt1]DALDA was administered intrathecally. Tail-flick latencies were measured prior to the administration of any drug (baseline latency) and at the time of peak analgesia (response latency). Naloxone hydrochloride at 82.5 nmol or saline was administered intrathecally 10 min prior to the second tail-flick measurement (n = 4 for each group).

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Baseline Latencies</th>
<th>Response Latencies</th>
</tr>
</thead>
<tbody>
<tr>
<td>MOR + saline</td>
<td>2.78 ± 0.50</td>
<td>7 (cut-off latency)*</td>
</tr>
<tr>
<td>DALDA + saline</td>
<td>2.63 ± 0.15</td>
<td>7 (cut-off latency)*</td>
</tr>
<tr>
<td>[Dmt1]DALDA + saline</td>
<td>2.83 ± 0.17</td>
<td>7 (cut-off latency)*</td>
</tr>
<tr>
<td>MOR + naloxone</td>
<td>3.08 ± 0.31</td>
<td>3.13 ± 0.41</td>
</tr>
<tr>
<td>DALDA + naloxone</td>
<td>2.65 ± 0.17</td>
<td>2.90 ± 0.43</td>
</tr>
<tr>
<td>[Dmt1]DALDA + naloxone</td>
<td>2.88 ± 0.10</td>
<td>2.68 ± 0.15</td>
</tr>
</tbody>
</table>

*Significantly different from baseline (p < 0.05).
had a minimum VE value lower than the critical level (a “low minimum VE”) (Table 4). Furthermore, one animal of the lower dose MOR group and one animal of the higher dose [Dmt1]DALDA group also had a low minimum VE, although the mean values of the groups were not significantly different from the saline group. No animal in the lower dose [Dmt1]DALDA group showed a low minimum VE. The timing of the occurrence of low minimum VE for each animal was between 3 and 5 h after administration for all groups.

Study 5: Effects of α2-Adrenergic Blockade. Yohimbine (100 μg) alone had no effect on tail-flick latency at any time compared with baseline value (data not shown). The addition of yohimbine (100 μg) significantly attenuated the antinociceptive effect of [Dmt1]DALDA (1.1 pmol) (Fig. 4), but not DALDA (240 pmol) (Fig. 5).

Behavioral Effects. Rigidity of the caudal part of the body was observed in each animal that received the higher dose (see study 3) of MOR, DALDA, or [Dmt1]DALDA. Four rats in the group that was given the higher dose of DALDA and had a minimum VE less than 60% of baseline (Fig. 3) showed sedative effects that coincided with the period of respiratory depression. During this period, normal activity was markedly suppressed in these animals and they could not negotiate a 60° mesh (Shimoyama et al., 1997b). However, they retained their righting reflex. No overt sedative effects were observed in animals of other groups.

Inhibition of NE and 5-HT Uptake in Spinal Cord Synaptosomes. DALDA, [Dmt1]DALDA, and MOR all inhibited [3H]NE uptake in a dose-dependent manner (Fig. 6). The IC50 for inhibition of [3H]NE uptake was 4.1 μM (2.5–6.7) for [Dmt1]DALDA, 54 μM (28–107) for DALDA, and 870 μM (197–3832) for MOR. At a dose of 10−4 M, [Dmt1]DALDA inhibited [3H]NE uptake by 80.6 ± 1.5%. Neither DALDA nor [Dmt1]DALDA had any effect on 5-HT uptake in spinal cord synaptosomes (data not shown).

Discussion

In the present study, we demonstrated that intrathecal administration of DALDA and [Dmt1]DALDA produce dose-dependent antinociceptive effects that parallel the effects of intrathecal morphine. The potency ratios of the antinociceptive effect of the drugs were MOR:DALDA:[Dmt1]DALDA = 1:14:3000, with [Dmt1]DALDA showing extraordinary potency compared with morphine. The antinociceptive effects of DALDA and [Dmt1]DALDA were reversed by intrathecal administration of the opioid antagonist naloxone. This indicates that, although the peptide analogs are highly charged, they can penetrate through the pia mater and into the spinal cord to act on spinal opioid receptors. The time to peak effect was longer for DALDA and [Dmt1]DALDA compared with morphine, reflecting their slower rate of penetration into the spinal cord.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Dose</th>
<th>Total No. of Rats</th>
<th>No. of Rats with Low Minimum VE</th>
<th>Time of Occurrence of Low Minimum VE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td></td>
<td>7</td>
<td>0</td>
<td>4 h after administration</td>
</tr>
<tr>
<td>Morphine</td>
<td>10 nmol</td>
<td>6</td>
<td>1</td>
<td>4 h after administration</td>
</tr>
<tr>
<td>DALDA</td>
<td>0.7 nmol</td>
<td>6</td>
<td>4</td>
<td>3–5 h after administration</td>
</tr>
<tr>
<td></td>
<td>7 nmol</td>
<td>8</td>
<td>4</td>
<td>3–5 h after administration</td>
</tr>
<tr>
<td>[Dmt1]DALDA</td>
<td>3.4 pmol</td>
<td>6</td>
<td>0</td>
<td>3–5 h after administration</td>
</tr>
<tr>
<td></td>
<td>34 pmol</td>
<td>7</td>
<td>1</td>
<td>3 h after administration</td>
</tr>
</tbody>
</table>

Fig. 4. Effect of yohimbine on the antinociceptive effects of [Dmt1]DALDA. [Dmt1]DALDA (1.1 pmol) alone and DALDA (240 pmol) and yohimbine (100 μg), or yohimbine (100 μg) alone were given intrathecally to rats. Tail-flick latencies were measured before and every 20 min after drug administration. Yohimbine significantly attenuated the antinociceptive effect of [Dmt1]DALDA (p < 0.05, two-way ANOVA) while not having any effect on tail-flick latency by itself.

Fig. 5. Effect of yohimbine on the antinociceptive effects of DALDA. DALDA (240 pmol) alone, DALDA (240 pmol) and yohimbine (100 μg), or yohimbine (100 μg) alone were given intrathecally to rats. Tail-flick latencies were measured before and every 20 min after drug administration. The antinociceptive effect of DALDA was not significantly attenuated by yohimbine.

TABLE 4

The occurrence of respiratory depression after intrathecal administration of MOR, DALDA, and [Dmt1]DALDA

Doses of 3 times the ED50 value and 30 times the ED50 value (antinociceptive) of each drug were administered. A minimum VE value (see Materials and Methods) less than the mean − (2 × S.D.) of the minimum VE value of the saline group was considered a “low minimum VE”.
their in vitro binding affinity and potency in the isolated GPI [Dmt1]DALDA was unexpected because it is slightly less (Stone et al., 1997). The longer duration of action due to rapid breakdown in the spinal cord, due to their high hydrophilicity. Their resistance against enzymatic degradation further enhances their duration of action. Both peptides were found to be completely stable when incubated in sheep blood for 2 h at 37°C (H. H. Szeto, J. L. Lovelace, and D. M. Desiderio, unpublished data). In contrast, endomorphin-1 and -2 were reported to produce 5-HT inhibition further enhances their duration of action. Both peptides were found to be completely stable when incubated in sheep blood for 2 h at 37°C (H. H. Szeto, J. L. Lovelace, and D. M. Desiderio, unpublished data). In contrast, endomorphin-1 and -2 were reported to produce only a short-lasting antinociceptive effect after intrathecal administration due to rapid breakdown in the spinal cord (Stone et al., 1997). The longer duration of action of [Dmt1]DALDA was unexpected because it is slightly less polar than DALDA due to the two additional methyl groups in the Dmt residue. This difference in response duration may be due to different receptor binding kinetics, with [Dmt1]DALDA having a slower $k_{off}$ rate than DALDA.

Antinociceptivity of $\mu$-opioid agonists generally parallels their in vitro binding affinity and potency in the isolated GPI assay. However, the extraordinary potency of [Dmt1]DALDA after intrathecal administration cannot be explained by its affinity and potency at the $\mu$-opioid receptor. The affinity of [Dmt1]DALDA for the $\mu$-opioid receptor is only 7-fold greater than morphine and its potency in the in vitro functional assay (GPI) is only 20-fold greater than morphine. Yet intrathecal [Dmt1]DALDA is 3000-fold more potent than morphine. The potency of intrathecal DALDA is also greater than would be predicted based on its binding affinity and potency in the GPI assay. A synergistic interaction between $\alpha_2$-adrenergic receptors and 5-HT receptors with $\mu$-opioid receptors in the spinal cord has been recognized for many years (Larson and Takemori, 1977; Yaksh and Reddy, 1981; Loomis et al., 1987; Wilcox et al., 1987). Concurrent administration of $\alpha_2$-adrenergic agonists or NE uptake inhibitors significantly potentiates the antinociceptive potency of $\mu$-opioid agonists. Co-administration of subthreshold doses of morphine and desipramine (NE uptake inhibitor) or morphine and serotonin resulted in pronounced antinociception in rats (Reimann et al., 1999). In this study, we found that DALDA and [Dmt1]DALDA inhibited the uptake of NE in spinal cord synaptosomes in a dose-dependent manner, with IC50 values of 54 and 4 $\mu$M, respectively. Neither peptide analog affected 5-HT uptake in spinal cord synaptosomes. Although morphine can also inhibit NE uptake in spinal cord synaptosomes, it was at least 16-fold less potent than DALDA and 212-fold less potent than [Dmt1]DALDA. The resultant increase in extracellular NE may result in a synergistic interaction between $\alpha_2$-adrenergic receptors and $\mu$-opioid receptors. Indeed, the antinociceptive response to [Dmt1]DALDA was significantly attenuated by pretreatment with intrathecal yohimbine, an $\alpha_2$-adrenergic antagonist. Inhibition of NE uptake did not appear to contribute significantly to the antinociceptive action of DALDA and this is likely due to the lower potency of DALDA.

Thus, [Dmt1]DALDA may be regarded as a drug with dual actions, and its antinociceptive potency is better described by considering not only its affinity and potency at $\mu$-opioid receptors but also its potency at inhibiting NE uptake (Table 5). The 10-fold higher affinity of [Dmt1]DALDA for the $\mu$-opioid receptor, and its 10-fold higher potency in inhibiting NE uptake, may explain the 200-fold higher antinociceptive potency of [Dmt1]DALDA compared with DALDA after intrathecal administration. Similarly, the extraordinary potency of [Dmt1]DALDA compared with morphine may be accounted for by its 7-fold higher affinity for the $\mu$-opioid receptor and more than 100-fold higher potency in inhibiting NE uptake.

A similar combination of $\mu$-opioid activity and monoamine uptake-inhibiting activity was previously described for tramadol, an orally active synthetic analgesic that has been reported to be devoid of abuse liability and respiratory depression (Lehmann et al., 1990; Franceschini et al., 1999). Despite its 1000-fold lower affinity for the $\mu$-opioid receptor compared with morphine (2.1 $\mu$M), tramadol has been found to induce antinociception with a potency only 5- to 10-fold less than that of morphine. (±)-Tramadol was found to inhibit synaptosomal NE and 5-HT uptake with K1 values of 0.8 and 1.0 $\mu$M, respectively, and its antinociceptive activity was attenuated by both the $\alpha_2$-antagonist yohimbine and the

![Fig. 6](image)

**TABLE 5**

Relative binding affinity and potency at $\mu$-opioid receptor, NE uptake, and intrathecal potency

<table>
<thead>
<tr>
<th></th>
<th>MOR</th>
<th>DALDA</th>
<th>[Dmt1]DALDA</th>
<th>Tramadol*</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\mu$-Opioid binding affinity</td>
<td>1</td>
<td>0.6</td>
<td>7</td>
<td>0.001</td>
</tr>
<tr>
<td>Potency in GPI assay</td>
<td>1</td>
<td>0.1</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>Inhibition of NE uptake</td>
<td>1</td>
<td>16</td>
<td>212</td>
<td>1000</td>
</tr>
<tr>
<td>Intrathecal potency</td>
<td>1</td>
<td>14</td>
<td>3160</td>
<td>0.2</td>
</tr>
</tbody>
</table>

* Derived from Codd et al. (1995).
serotonin antagonist ritanserin (Codd et al., 1995). The authors of that article found that although inclusion of 5-HT uptake inhibition significantly improved the correlation between in vitro activity and in vivo antinociceptive potency for (±)-tramadol, consideration of NE uptake inhibition did not improve the correlation. Interestingly, neither DALDA nor [Dmt¹]DALDA had any effect on 5-HT uptake. However, their 1000- and 10,000-fold higher affinity for the µ-opioid receptor can readily explain their significantly higher antinociceptive potency compared with (±)-tramadol (Table 5).

Respiratory depression is the most disturbing side effect associated with intrathecal administration of opioid drugs. The onset of respiratory depression is generally delayed relative to the analgesic response and is related to the time required for rostral distribution of the drug to the medullary respiratory centers. Indeed, late respiratory depression has been reported in patients and human volunteers after receiving intrathecal morphine (Davies et al., 1980; Bailey et al., 1993), and rostral redistribution of morphine has been demonstrated in animals (Payne and Inturrisi, 1985). Distribution of hydrophilic drugs into the systemic circulation is unlikely to result in significant plasma drug levels to produce respiratory depression. In the present study, rats that received a high dose of morphine (30 times ED₅₀ value) exhibited a significant depression of minute ventilation compared with the group that received only saline. Respiratory depression occurred at 3 h after administration of morphine. Deposition of minute ventilation was also observed in rats that received intrathecal DALDA. With DALDA, both the lower dose (3 times ED₅₀ value) and higher dose (30 times ED₅₀ value) produced depression in minute ventilation, suggesting a greater propensity of DALDA to produce respiratory depression than morphine. This may be explained by the higher polarity of DALDA, which probably resulted in a greater amount of the drug remaining in the CSF to be redistributed rostrally. The peak respiratory effects were again observed at 3 to 5 h after administration, indicating that the rostral redistribution of morphine and DALDA occurs in a similar manner and the rate is only determined by bulk flow of CSF. On the other hand, no significant depression of minute ventilation was seen after either low or high dose of [Dmt¹]DALDA. Receptor selectivity cannot explain the difference in respiratory depression between DALDA and [Dmt¹]DALDA since they are both ~10,000-fold more selective for the µ-opioid receptor compared with the δ-opioid receptor. A more likely explanation may be its combined action at the µ-opioid receptor and NE transporter that allows for a lower dose of [Dmt¹]DALDA to be used and thus greatly reducing the incidence of opioid-induced respiratory depression. This has been the rationale behind the combined use of low doses of opioid, noradrenergic, and serotoninergic drugs to maximize pain control and minimize side effects of each agent (Reimann et al., 1999).

In conclusion, the highly charged dermorphin analogs DALDA and [Dmt¹]DALDA, when given intrathecally, can penetrate the spinal cord to produce long-lasting, dose-dependent antinociceptive effects. Their metabolic stability and polar nature account for their long duration of action, whereas the ability of [Dmt¹]DALDA to inhibit NE uptake synergizes with its action at the µ-opioid receptor and greatly increases its analgesic potency and reduces respiratory depression. Because of its extraordinary antinociceptive potency, long duration of action, and minimal respiratory depression, [Dmt¹]DALDA may be useful as an intrathecal opioid analog in patients.

Acknowledgments

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References


Schiller PW, Lipton A, Horrobin DF and Bodansky M (1978) Unsulfated C-terminal 8-amino-2, 10, 10,000-fold more selective µ-opioid receptor and greatly reduces respiratory depression. Because of its extraordinary antinociceptive potency, long duration of action, and minimal respiratory depression, [Dmt¹]DALDA may be useful as an intrathecal opioid analog in patients.

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