Endogenous Opioid Peptides Contribute to Antinociceptive Potency of Intrathecal [Dmt]$^{1}$DALDA

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ABSTRACT

[Dmt]$^{1}$DALDA (H-Dmt-d-Arg-Phe-Lys-NH$_2$; Dmt = 2′,6′-dimethyltyrosine) is a dermorphin analog that shows high affinity and selectivity for the μ opioid receptor. The intrathecal potency of [Dmt]$^{1}$DALDA far exceeded its affinity at μ receptors and suggests that other mechanisms must be involved in its action in the spinal cord. The affinity and selectivity of [Dmt]$^{1}$DALDA was determined using cell membranes expressing cloned human μ, δ, and κ opioid receptors. Competitive displacement binding with [3H][Dmt]$^{1}$DALDA, [3H]DPDPE (H-Tyr-D-Ala-Gly-NMePhe-Gly-ol) (Riba et al., 2002), and [3H]U69,593 ([5α,7α,8β]-(+)-N-methyl-N-[7-[1-pyrrolidinyl]-1-oxaspiro[4.5]dec-8-yl]-benzeneacetamide) revealed K$_i$ of 156 ± 26 pm for μ opioid receptor (MOR), 1.67 ± 0.04 μM for δ opioid receptor (DOR), and K$_i$ of 4.4 ± 1.7 nM for κ opioid receptor (KOR), respectively. [Dmt]$^{1}$DALDA increased guanosine 5′-O-(3-[35S]thiotriphosphate) binding in MOR, DOR, and KOR membranes, with EC$_{50}$ being 17 (8.8–33) nM, 2 (1.2–3.2) μM, and 124 (15–1000) nM, respectively. Intrathecal [Dmt]$^{1}$DALDA inhibited the tail-flick response in mice with ED$_{50}$ = 1.22 (0.59–2.34) pmol. Intrathecal administration of an antiserum against dynorphin A(1-17) or [Met]$^{6}$enkephalin significantly attenuated the response to i.t. [Dmt]$^{1}$DALDA, resulting in ED$_{50}$ of 6.2 (3.6–12.6) pmol and 6.6 (3.5–19.6) pmol, respectively. Neither antisera had any effect on the response to i.t. morphine. Intracerebroventricular (i.c.v.) [Dmt]$^{1}$DALDA was not affected by previous i.c.v. administration of anti-Dyn or anti-ME. Pretreatment with norbinaltorphimine or naltrindol also attenuated the antinociceptive response to i.t., but not i.c.v., [Dmt]$^{1}$DALDA. These data suggest that i.t. [Dmt]$^{1}$DALDA causes the release of dynorphin and [Met]$^{6}$enkephalin-like substances that act at κ and δ receptors, respectively, to contribute to the extraordinary potency of [Dmt]$^{1}$DALDA.

Of the three subtypes of opioid receptors (μ, δ, and κ), μ appears to be most important in analgesia. [Dmt]$^{1}$DALDA (H-Dmt-d-Arg-Phe-Lys-NH$_2$; Dmt = 2′,6′-dimethyltyrosine) is a dermorphin analog with extraordinary affinity (K$_a$ ~150 pM) and selectivity for μ receptors (Schiller et al., 2000). [Dmt]$^{1}$DALDA was reported to be ~14,000 and ~150 times more selective for μ than δ and κ, respectively (Schiller et al., 2000). Surprisingly, the in vivo analgesic potency of [Dmt]$^{1}$DALDA far exceeded its affinity and potency at μ receptors. Compared with morphine, [Dmt]$^{1}$DALDA was 30 to 200 times more potent after intracerebroventricular (i.c.v.) administration, and 1,000 to 5,000 times more potent after intrathecal (i.t.) administration (Neilan et al., 2001; Shimoyama et al., 2001; Riba et al., 2002; Zhao et al., 2002). In contrast, the affinity of [Dmt]$^{1}$DALDA for the μ receptor was estimated to be only 7-fold greater compared with morphine (Schiller et al., 2000).

The extraordinary potency of intrathecal [Dmt]$^{1}$DALDA suggested that mechanisms other than activation of μ receptors must be involved in its analgesic action in the spinal cord. Previous studies using selective μ, δ, and κ antagonists concluded that the antinociceptive action of [Dmt]$^{1}$DALDA was mediated via μ receptors, and that [Dmt]$^{1}$DALDA was interacting with the same population of spinal μ receptors as DAMGO (H-Tyr-d-Ala-Gly-NMePhe-Gly-ol) (Riba et al., 2002). However, [Dmt]$^{1}$DALDA exhibited little to no cross-tolerance in morphine-tolerant animals, which led to the suggestion that [Dmt]$^{1}$DALDA and morphine may be acting at different subtypes of μ receptors (Neilan et al., 2001; Riba et al., 2002). Differences in response to [Dmt]$^{1}$DALDA and morphine in different mouse strains and with antisense oligodeoxynucleotides targeting against specific exons of the mouse μ opioid receptor gene support the suggestion that [Dmt]$^{1}$DALDA and morphine act at different subtypes of μ receptors (Neilan et al., 2001). However, repeated systemic administration of [Dmt]$^{1}$DALDA...
resulted in profound tolerance to both [Dmt\(^1\)]DALDA and morphine in the spinal cord, but with little supraspinal tolerance (Zhao et al., 2002). This recent finding raises the possibility that [Dmt\(^1\)]DALDA might act via different mechanisms in the spinal cord versus brain.

It was recently reported that endomorphin-1 and endomorphin-2, two opioid peptides isolated from mammalian brain, act via different mechanisms after i.t. administration. Both endomorphin peptides demonstrate high affinity and selectivity for \(\mu\) receptors in radioligand binding assays (Zadina et al., 1997). However, an additional component of the antinociceptive response to i.t. endomorphin-2 is due to the release of dynorphin A(1-17) and [Met\(^5\)]enkephalin which subsequently acts on \(\kappa\) and \(\delta\) receptors, respectively, in the spinal cord (Ohsawa et al., 2001; Sakurada et al., 2001). Activation of \(\delta\) and \(\kappa\) receptors can potentiate the action of \(\mu\) opioid agonists in the spinal cord (Porreca et al., 1992; He and Lee, 1998). In this study, we examined whether spinal dynorphin and [Met\(^5\)]enkephalin may play a role in the spinal action of [Dmt\(^1\)]DALDA.

### Materials and Methods

**Animals.** Male CD-1 mice (25–30 g) were purchased from Charles River Laboratories (Wilmington, MA). Animals were housed in a temperature-controlled room maintained on a 12-h light/dark cycle. Food and water were available ad libitum until the time of the experiment. All experiments were conducted in accordance with the ethical guidelines of the International Association for the Study of Pain and approved by the Institution for the Care and Use of Animals at Weill Medical College of Cornell University.

**Drugs and Chemicals.** [Dmt\(^1\)]DALDA and [\(^{3}H\)][Dmt\(^1\)]DALDA (47 Ci/mmol) were synthesized by Dr. Peter W. Schiller (Clinical Research Institute of Montreal, QC, Canada) according to methods described previously (Schiller et al., 1989, 2000; Zhao et al., 2002). [\(^{3}H\)]DPDPE (\(H\)-Tyr-i-tyr-Pen-Gly-Phe-n-Pen; 42 Ci/mmol), [\(^{3}H\)]enkephalin (\(\alpha\)-Ala\(^3\) d)eltorphin II, and morphine sulfate were provided by the National Institute on Drug Abuse (Rockville, MD). [\(^{3}H\]]U69,593 ([5a,7a,8b(\(\pm\))]-N-methyl-N-(5-\(\gamma\)-pyrrolidinyl)-1-oxaspiro[4,5]dec-8-yl)benzeneacetamide) ([9 C/mmol]) and [\(^{35}S\)]GTP\(\gamma\)S (1000–1200 Ci/mmol) were purchased from Amersham Biosciences, Inc. (Piscataway, NJ). All other drugs and chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

**Antisera.** Rabbit antisera against dynorphin A(1-17) and [Met\(^5\)]enkephalin were obtained from Peninsula Laboratories/Bachem (San Carlos, CA). According to the manufacturer, the antisemum against dynorphin A(1-17) showed 0.43% cross-reactivity with dynorphin A(1-13), but does not cross-react with dynorphin A(1-8), dynorphin B, or [Leu\(^{5}\)]enkephalin. The antisemum against [Met\(^5\)]enkephalin showed cross-reaction against [Met\(^5\)]enkephalin-Arg-Phe (0.1%), [Leu\(^{5}\)]enkephalin (3%), and \(\beta\)-endorphin (0.1%), but not against dynorphin A(1-17).

**MOR, DOR, and KOR Membranes.** Membranes prepared from either CHO-K1 cells transfected with hMOR or hDOR, or HEK293 cells transfected with hKOR were purchased from PerkinElmer Life Sciences (Boston, MA).

**Radioligand Binding Assay.** The binding affinity of [Dmt\(^1\)]DALDA to \(\mu\), \(\delta\), and \(\kappa\) receptors was determined by competitive displacement binding using membranes expressing hMOR, hDOR, and hKOR, as described above. All binding assays were carried out using 400 \(\mu\)g of protein of membranes. For binding to \(\mu\) receptors, hMOR membranes were incubated with 100 pM [\(^{3}H\)][Dmt\(^1\)]DALDA and graded concentrations of unlabeled [Dmt\(^1\)]DALDA for 60 min at 25°C. Nonspecific binding was determined using 1 \(\mu\)M [Dmt\(^1\)]DALDA. For binding to \(\delta\) receptors, hDOR membranes were incubated with 2 nM [\(^{3}H\)]DPDPE and graded concentrations of [Dmt\(^1\)]DALDA for 120 min at 25°C, and nonspecific binding was determined using 8 \(\mu\)M unlabeled DPDPE. For binding to \(\kappa\) receptors, hKOR membranes were incubated with 0.8 nM [\(^{3}H\]U69,593 and graded concentrations of unlabeled [Dmt\(^1\)]DALDA for 80 min at 25°C, and nonspecific binding was determined with 10 \(\mu\)M naloxone. Free radioligand was separated from bound radioligand by rapid filtration through GF/B filters (Brandel, Gaithersburg, MD) with a cell harvester (Brandel). Filters were washed three times with 3 ml of Tris-HCl buffer. Radioactivity was determined by liquid scintillation counting. All binding experiments were carried out in triplicate, and the results represent mean ± S.E. from four to six experiments. IC\(_{50}\) was determined from the displacement curves using nonlinear regression (GraphPad, San Diego, CA). K\(_v\) values were calculated from the IC\(_{50}\) values by means of the Cheng and Prusoff equation, \(K_v = IC_{50}/(1 + L/K_d)\), where L and K\(_d\) are the concentration and affinity of the radiolabeled ligand in the assay (Cheng and Prusoff, 1973). The K\(_d\) values for [\(^{3}H\)][Dmt\(^1\)]DALDA, [\(^{3}H\)]DPDPE, and [\(^{3}H\]U69,593 in the MOR, DOR, and KOR membranes were previously determined to be 154 ± 10 pM, 1.95 ± 0.20 nM, and 0.694 ± 0.08 nM, respectively (data not shown).

**[\(^{35}S\)]GTP\(\gamma\)S Binding Assay.** Activation of [\(^{35}S\)]GTP\(\gamma\)S binding by increasing concentrations of [Dmt\(^1\)]DALDA in hMOR, hDOR, and hKOR membranes was used to ascertain the potency and intrinsic activity of [Dmt\(^1\)]DALDA at \(\mu\), \(\delta\), and \(\kappa\) receptors, respectively. Aliquots of membrane homogenates (8 \(\mu\)g of protein) were incubated with 80 pM [\(^{35}S\)]GTP\(\gamma\)S and 30 \(\mu\)M GDP in 1 ml Tris buffer (50 mM Tris-HCl, 100 mM NaCl, 5 mM MgCl\(_2\), 1 mM dithiothreitol, 1 mM EDTA, 0.1% bovine serum albumin, pH 7.4) in the presence of varying concentrations of [Dmt\(^1\)]DALDA for 60 min at 30°C. Nonspecific binding was determined using 10 \(\mu\)M unlabeled GTP\(\gamma\)S. Free radioligand was separated from bound radioligand by rapid filtration. All experiments were carried out in triplicate, and the results represent data from four to six experiments. Potency (EC\(_{50}\)) and intrinsic activity (E\(_{max}\)) were determined using nonlinear regression (Graphpad). The EC\(_{50}\) values are presented with 95% confidence intervals.

**Drug Administration.** Drugs were administered i.t., i.c.v., or subcutaneously (s.c.) to mice. Intrathecal injection was carried out according to the method described by Hylden and Wilcox (1980). The needle (30-gauge) was inserted from the side of the L5 or L6 spinous process and the injection volume was 4 \(\mu\)l/mouse. For i.c.v. injections, mice were lightly anesthetized with isoflurane and an incision made over the scalp to expose the bregma. The injection (4 \(\mu\)l) was delivered 2 mm lateral and caudal to the bregma to a depth of 3 mm (Haley and McCormick, 1957). Groups of 10 to 20 mice were used for each dose and each mouse was only used once. Potency was determined at 30 min after i.t. and i.c.v. injection.

**Antisera and Antagonists Pretreatment.** Antiserum against dynorphin A(1-17) or [Met\(^5\)]enkephalin were administered either i.t. or i.c.v. in an injection volume of 4 \(\mu\)l, 30 min before [Dmt\(^1\)]DALDA administration. The \(\kappa\) antagonist, norbinaltorphimine (norBNI), was administered s.c. 24 h before [Dmt\(^1\)]DALDA. The \(\delta\) antagonist, naltriben, was administered s.c. 30 min before [Dmt\(^1\)]DALDA.

**Antinociceptive Assay.** The radiant heat tail-flick assay was used for antinociceptive tests in mice. The light intensity was adjusted such that the baseline latencies ranged between 2.5 and 3.5 s. To avoid tissue damage, a cut-off of 10 s was used. Antinociceptive activity for each animal was expressed as percent of maximal possible effect (%MPE) which was calculated as (P2 – P1)/(10 – P1) × 100, where P1 and P2 are predrug and postdrug response time, respectively. For dose-response analyses, the percentage of analgesic responders was calculated and the quantal dose-response curves analyzed using probit analysis (PharmTools Pro; McCary Group Inc., Elins Park, PA). Antinociception was defined as a latency response of greater than two times the baseline latency for an individual animal. Data are presented as ED\(_{50}\) with 95% confidence intervals. Statistical comparisons of dose-response curves were performed by analysis of variance with F-Statistic (PharmTools Pro).
Results

Radioligand Binding Assay. The affinity of [Dmt1]DALDA for μ, δ, and κ receptors was originally ascertained by displacement of [3H]DAMGO and [3H]DSLET binding from rat brain membranes, and displacement of [3H]U69,593 binding from guinea pig brain membranes, respectively (Schiller et al., 2000). Because brain tissues express more than one subtype of opioid receptors and the specificity of these radioligands are not absolute, we have evaluated the selectivity of [Dmt1]DALDA by determining its affinity in pure populations of μ, δ, and κ receptors. Competitive displacement binding resulted in $K_i$ values for hMOR membranes of 156 ± 26 pM ($n = 6$), 1.67 ± 0.04 μM for hDOR membranes ($n = 4$), and 4.4 ± 1.7 nM for hKOR membranes ($n = 4$). This resulted in a μ/δ selectivity of 10,700 and a μ/κ selectivity of 27.

[35S]GTPγS Binding. The functional activity of [Dmt1]DALDA was previously determined using the guinea pig ileum and mouse vas deferens assays (Schiller et al., 2000). Since both guinea pig ileum and mouse vas deferens contain more than one subtype of opioid receptors, we have compared the potency and intrinsic activity of [Dmt1]DALDA in [35S]GTPγS binding using hMOR, hDOR, and hKOR membranes. The results are summarized in Fig. 1. [Dmt1]DALDA increased [35S]GTPγS binding in all three membranes, with EC$_{50}$ values of 17 (8.8–33) nM for hMOR ($n = 4$), 2.0 (1.2–3.2) μM for hDOR ($n = 5$), and 124 (15–1000) nM for hKOR ($n = 4$).

Effects of Pretreatment with norBNI on i.t. Administration of [Dmt1]DALDA. To determine whether the κ opioid receptor plays a role in the intrathecal action of [Dmt1]DALDA, mice were pretreated with the κ antagonist, norBNI (13.6 μmol/kg, s.c., 24 h before i.t. administration of [Dmt1]DALDA (4.1 pmol). The antinociceptive response to i.t. [Dmt1]DALDA was significantly reduced in mice pretreated with norBNI (Fig. 2A) and the ED$_{50}$ of i.t. [Dmt1]DALDA was increased from 1.22 (0.59–2.34) pmol to 11.6 (3.09–44.8) pmol ($P < 0.05$) (Fig. 2B).

Effects of Pretreatment with norBNI on i.c.v Administration of [Dmt1]DALDA. Mice were pretreated with norBNI (13.6 μmol/kg, s.c.) 24 h before i.c.v. administration of [Dmt1]DALDA (10.2 pmol). Figure 3 shows that, in contrast to i.t. [Dmt1]DALDA, the antinociceptive response to i.c.v. [Dmt1]DALDA was not affected by norBNI.

Effects of Pretreatment with Antiserum against Dynorphin A(1-17) on Tail-Flick Inhibition induced by i.t. and i.c.v. Administration of [Dmt1]DALDA. To determine whether spinal dynorphin contributes to the antinociceptive response to i.t. and i.c.v. [Dmt1]DALDA in the absence and presence of norBNI. Mice were pretreated with norBNI (13.6 μmol/kg, s.c.) 24 h before i.t. or i.c.v. administration of [Dmt1]DALDA. Pretreatment with norBNI increased the ED$_{50}$ of i.t. [Dmt1]DALDA from 1.22 to 11.6 pmol ($P < 0.05$). Figure 3 shows that, in contrast to i.t. [Dmt1]DALDA, the antinociceptive response to i.c.v. [Dmt1]DALDA was not affected by norBNI.

Fig. 1. The stimulation of [35S]GTPγS binding by [Dmt1]DALDA to membranes prepared from CHO-K1 cells transfected with hMOR or hDOR, and HEK293 cells transfected with hKOR. Membranes were incubated with 50 μM [35S]GTPγS and 30 μM GDP in the presence of varying concentrations of [Dmt1]DALDA for 60 min at 30°C. Nonspecific binding was determined using 10 μM unlabeled GTPγS. [Dmt1]DALDA increased [35S]GTPγS binding in all three membranes, with EC$_{50}$ values of 17 (8.8–33) nM for MOR ($n = 4$), 2.0 (1.2–3.2) μM for DOR ($n = 5$), and 124 (15–1000) nM for KOR ($n = 4$).

Fig. 2. Effects of norBNI on tail-flick inhibition induced by i.t. [Dmt1]DALDA in CD-1 mice. A, Time course of antinociceptive response to i.t. [Dmt1]DALDA (4.1 pmol) in the absence and presence of norBNI. B, Dose-response curves for i.t. [Dmt1]DALDA in the absence and presence of norBNI. Pretreatment with norBNI increased the ED$_{50}$ of i.t. [Dmt1]DALDA from 1.22 to 11.6 pmol ($P < 0.05$).
dynorphin A(1-17) 30 min before i.t. administration of an ED_{50} dose of [Dmt^{1}]DALDA (4.1 pmol). Figure 4A shows the reduction in antinociceptive response to i.t. [Dmt^{1}]DALDA following i.t. dynorphin A(1-17) antiserum pretreatment. The maximal reduction in antinociceptive response (30\%MPE) was observed with 1:200 of the antiserum. Pretreatment with this dilution of dynorphin A(1-17) antiserum increased the ED_{50} of [Dmt^{1}]DALDA from 1.22 pmol to 6.2 (3.6–12.6) pmol (P < 0.05) (Fig. 4B). In contrast, i.c.v. pretreatment with the same dilution of dynorphin A(1-17) antiserum had no effect on the antinociceptive response to i.c.v. [Dmt^{1}]DALDA (10.2 pmol) (data not shown).

Effects of Pretreatment with Antiserum against [Met^{5}]Enkephalin on i.t. and i.c.v. Administration of [Dmt^{1}]DALDA. To determine whether [Met^{5}]enkephalin also contributes to the antinociceptive response to i.t. [Dmt^{1}]DALDA, groups of mice were pretreated i.t. with varying dilutions of [Met^{5}]enkephalin antiserum 30 min before i.t. administration of an ED_{50} dose of [Dmt^{1}]DALDA (4.1 pmol). This antiserum does not show cross-reactivity to dynorphin peptides. Figure 5A shows the reduction in antinociceptive response to i.t. [Dmt^{1}]DALDA following i.t. [Met^{5}]enkephalin antiserum pretreatment. The maximal reduction in antinociceptive response (30\%MPE) was observed with 1:50 dilution of the antiserum. Pretreatment with this dilution of [Met^{5}]enkephalin antiserum (1:50) increased the ED_{50} of [Dmt^{1}]DALDA from 1.22 pmol to 6.6 (3.5–19.6) pmol (P < 0.05) (Fig. 5B). In contrast, i.c.v. pretreatment with the same dilution of [Met^{5}]enkephalin antiserum had no effect on the antinociceptive response to i.c.v. [Dmt^{1}]DALDA (10.2 pmol) (data not shown).

Effects of Naltriben Pretreatment on i.t. [Dmt^{1}]DALDA. To establish a role for the \( \delta \) opioid receptor in the spinal action of [Dmt^{1}]DALDA, mice were pretreated with naltriben (3 mg/kg, s.c.) 30 min before administration of [Dmt^{1}]DALDA (4.1 pmol, i.t.). This dose of naltriben was chosen because it significantly reduced the antinociceptive response to i.t. deltorphin and increased its ED_{50} from 7.42 (4.0–15.2) to 20.8 (12.1–40.9) nmol (Fig. 6B). This dose of naltriben also significantly reduced the antinociceptive response to i.t. [Dmt^{1}]DALDA (Fig. 6B).

Effects of i.t. Pretreatment with Antisera against Dynorphin A(1-17) or [Met^{5}]Enkephalin on i.t. Morphine. To determine whether the additional contribution of dynorphin and [Met^{5}]enkephalin on spinal antinociception was unique to [Dmt^{1}]DALDA, we also examined the effect of dynorphin antiserum and [Met^{5}]enkephalin antiserum on i.t. morphine.
Morphine. Mice were administered an ED$_{80}$ dose of morphine (2.63 nmol) 30 min after i.t. administration of either dynorphin antiserum (1:200) or [Met$^5$]enkephalin antiserum (1:50). Neither antiserum had any effect on the antinociceptive response to i.t. morphine (Fig. 7).

Effects of Pretreatment with Naloxonazine on i.t. [Dmt$^1$]DALDA. The different responses of [Dmt$^1$]DALDA and morphine to dynorphin antiserum and [Met$^5$]enkephalin antiserum suggested the possibility that they may be acting on different subtypes of $\mu$ opioid receptor. Pretreatment with naloxonazine ($\mu_1$ antagonist; 48.3 $\mu$mol/kg, s.c.) 24 h before did not significantly alter the ED$_{50}$ to i.t. or i.c.v. [Dmt$^1$]DALDA (data not shown).
Endogenous Opioid Peptides and Intrathecal [Dmt¹]DALDA

Discussion

Previous studies revealed that the intrathecal potency of [Dmt¹]DALDA far exceeded its affinity and potency at the µ opioid receptor. This discrepancy led us to postulate that additional mechanisms other than activation of µ opioid receptor must be involved in the spinal action of [Dmt¹]DALDA. Our present study confirms the extraordinary selectivity of [Dmt¹]DALDA for the µ opioid receptor. In the original study, the selectivity of [Dmt¹]DALDA was based on displacement of [³H]DAMGO and [³H]DSLET binding to mouse brain membranes and displacement of [³H]U69,593 binding to guinea pig brain membranes (Schiller et al., 2000). With the availability of pure populations of transfected MOR, DOR, and KOR and the recent synthesis of [³H][Dmt¹]DALDA (Zhao et al., 2002), we were able to more accurately determine the affinity of [Dmt¹]DALDA for the three opioid receptor subtypes. The affinity of [Dmt¹]DALDA, as determined from saturation binding with [³H][Dmt¹]DALDA and MOR membranes, was found to be 154 ± 10 pM, and this is in excellent agreement with the Kᵢ reported previously (143 pM) (Schiller et al., 2000). The µ/δ selectivity of [Dmt¹]DALDA was found to be ~10,000, which is also similar to the earlier estimate (14,700) obtained from brain membranes (Schiller et al., 2000) and is similar to that reported for endomorphin-1 and endomorphin-2 (Zadina et al., 1997). [Dmt¹]DALDA has modest affinity for KOR, and the MOR/KOR selectivity determined using pure receptor populations (27) is less than that previously determined in mouse brain membranes with selective radioligands (156) (Schiller et al., 2000).

The present study provides the first report on the potency and intrinsic activity of [Dmt¹]DALDA at the three opioid receptors using GTPγS binding as a measure of G protein activation. [Dmt¹]DALDA stimulated [³⁵S]GTPγS binding to membranes expressing MOR, DOR and KOR in a concentration-dependent manner. The maximal stimulation of [³⁵S]GTPγS binding by [Dmt¹]DALDA was 90% compared with DAMGO at hMOR, 123% compared with deltorphin II at hDOR, and 46% compared with U50,488H ((trans-±)-3,4-dichloro-N-methyl-[2-(1-pyrrolidinyl)-cyclohexyl] benzenac -etamide) at hKOR (G.-M. Zhao and H. H. Szeto, unpublished data). These data suggest that [Dmt¹]DALDA is a full agonist at hMOR and hDOR, but a partial agonist at hKOR.

The involvement of δ and κ opioid receptors in the spinal action of [Dmt¹]DALDA is most likely not due to direct action of [Dmt¹]DALDA on these two receptors, especially when the same antagonist treatment had no effect on supraspinal [Dmt¹]DALDA, and the µ/δ selectivity of [Dmt¹]DALDA is in excess of 10,000. We propose that [Dmt¹]DALDA causes the release of dynorphin-like and [Met⁵]enkephalin-like substances in the spinal cord that can then subsequently act on κ and δ receptors, respectively, to contribute to the overall antinociceptive action of intrathecal [Dmt¹]DALDA. This idea is supported by the finding that the antinociceptive response to intrathecal [Dmt¹]DALDA was significantly attenuated by intrathecal administration of antisera to dynorphin A(1-17) or [Met⁵]enkephalin. The dynorphin antisera used in the study was raised against dynorphin A(1-17), and shows only 0.43% cross-reactivity against dynorphin A(1-13), and none against [Leu⁵]enkephalin or dynorphin B. Likewise, the [Met⁵]enkephalin antisera only has 2.8% cross-reactivity against [Leu⁵]enkephalin and none against dynorphin A(1-17). These antisera had no effect on the antinociceptive response to intrathecal morphine, and i.c.v. administration of these antisera had no effect on supraspinal [Dmt¹]DALDA. This is consistent with the lack of effect of norBNI and naltrindol (another δ antagonist) had no effect on supraspinal [Dmt¹]DALDA; however, their effect on spinal [Dmt¹]DALDA was not examined (Neilan et al., 2001).

The enkephalin peptides mediate antinociception supraspinally and spinally by interacting with δ₁ and δ₂ receptors, respectively (Takemori and Portoghese, 1993). In our study, the response to i.t. [Dmt¹]DALDA was attenuated by pretreatment with naltriben on supraspinal [Dmt¹]DALDA. It was also noted in an earlier paper that norBNI and naltrindol (another δ antagonist) had no effect on supraspinal [Dmt¹]DALDA; however, their effect on spinal [Dmt¹]DALDA was not examined (Neilan et al., 2001).

The role of dynorphin in pain modulation is highly controversial. Whereas low doses of dynorphin produce antinociception, higher doses produce a long-lasting allodynia (Vanderah et al., 1996; Laughlin et al., 1997). Many studies support an antinociceptive function of dynorphin in the spinal cord by negatively modulating transmission of nociceptive information. Dynorphin can inhibit substance P release (Zachariou and Goldstein, 1997) and inhibit synaptic transmission of nociceptive neurons in the spinal cord (Randic et al., 1995) via activation of κ receptors. On the other hand, dynorphin can exert pronociceptive effects via nonopiod
mechanisms that involve N-methyl-d-aspartate receptors (Bakshi et al., 1992; Laughlin et al., 1997; Vanderah et al., 2000). Recent data obtained with dream mice clearly support an antinociceptive role for endogenous dynorphin. DREAM (downstream regulatory element antagonist modular) is a putative transcriptional repressor for the dynorphin gene (Carrión et al., 1999). Mice lacking DREAM had elevated levels of prodynorphin mRNA and dynorphin A peptides in the spinal cord and displayed reduced responses in models of acute thermal, mechanical, and visceral pain (Cheng et al., 2002).

Activation of spinal release of dynorphin was reported to contribute to the intrathecal action of endomorphin-2, but not endomorphin-1 (Ohsawa et al., 2001; Sakurada et al., 2001). The antinociceptive response to intrathecal endomorphin-2 was blocked by intrathecal pretreatment with antisense against dynorphin A(1-17) or norBNI. However, spinal dynorphin does not appear to play a role in the spinal action of endomorphin-1 nor DAMGO (Sakurada et al., 2001). We also found no effect of dynorphin antisense or norBNI on intrathecal morphine action. As the endomorphins and DAMGO are also highly selective μ agonists, these findings suggest that only certain μ agonists can elicit the release of dynorphin from the spinal cord. There is less evidence to support a role for endogenous enkephalins in the spinal action of other μ agonists. It was reported that [Met]$^3$enkephalin antisense blocked the effect of intrathecal endomorphin-2 in one study (Sakurada et al., 2001) but not in another (Ohsawa et al., 2001).

It has been proposed that these diverse μ agonists may activate different subtypes of μ receptors in the spinal cord (Sakurada et al., 1999, 2000). Autoradiographic studies have demonstrated the presence of both μ1 and μ2 receptors in the brain and spinal cord (Moskowitz and Goodman, 1985). Pretreatment with naloxonazine, the μ antagonist, partially blocked the action of intrathecal endomorphin-2 but not endomorphin-1 or DAMGO, suggesting that activation of μ1 receptors in the spinal cord leads to dynorphin release (Sakurada et al., 1999, 2001). In another study, however, the authors reported that the same dose of naloxonazine blocked all three agonists, although endomorphin-2 was more sensitive compared with endomorphin-1 and DAMGO (Sakurada et al., 2000). In general, available evidence suggests that μ1 is involved in supraspinal but not spinal analgesia. In the present study, naloxonazine had no significant effect on ei-

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