Methocinnamox Is a Potent, Long-Lasting, and Selective Antagonist of Morphine-Mediated Antinociception in the Mouse: Comparison with Clocinnamox, β-Funaltrexamine, and β-Chlornaltrexamine

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ABSTRACT

The irreversible μ-opioid antagonists β-funaltrexamine (β-FNA) and β-chlornaltrexamine (β-CNA) are important pharmacological tools but have a κ-opioid activity and, in the latter case, low selectivity. This work examines whether clocinnamox (C-CAM) and the newer analog, methocinnamox (M-CAM), represent improved long-lasting antagonists for examining μ-opioid-mediated effects in vivo. β-FNA, β-CNA, C-CAM, and M-CAM were compared after systemic administration in mice and in vitro. β-FNA and β-CNA were effective agonists in the writhing assay, reversible by the κ-opioid antagonist norbinaltorphimine. Neither C-CAM nor M-CAM had agonist activity in vivo. M-CAM was devoid of agonist action at cloned opioid receptors. All four compounds depressed the dose-effect curve for the μ-opioid morphine in the warm-water tail-withdrawal test 1 h after administration; at 48 h, recovery was evident. In the writhing assay, the dose-effect curve for morphine was shifted in a parallel fashion in the order M-CAM > C-CAM > β-CNA > β-FNA. In comparison with their ability to shift the dose-effect curve for bremazocine (κ) and BW373U86 (δ), β-CNA was the least μ-selective, followed by C-CAM < β-FNA < M-CAM. M-CAM (1.8 mg/kg) produced a 74-fold increase in the ED50 of morphine while showing no effect on bremazocine or BW373U86 dose-response curves. In binding assays, C-CAM and M-CAM were 8-fold selective for μ-receptors, whereas β-FNA and β-CNA were μ/δ, but not μ/κ, selective. However, ex vivo binding assays confirmed the μ-opioid selectivity of M-CAM. M-CAM is thus a potent, long-lasting, and specific antagonist at μ-receptors in vivo that lacks confounding agonist actions.

The role of the μ-receptor in opioid pharmacology in the mouse is best studied in knock-out animals (Matthes et al., 1996). However, in other species, it is necessary to provide complete and long-lasting blockade of the μ-opioid receptor to undertake similar studies. Development of the irreversible opioid antagonists β-chlornaltrexamine (β-CNA) and β-funaltrexamine (β-FNA; Takemori and Portoghese, 1985) and the long-lasting, but nonalkylating, antagonist clocinnamox (C-CAM; Comer et al., 1992; Fig. 1) provided the opportunity to study opioid agonists in the absence of a particular receptor type (Takemori and Portoghese, 1987), the relative efficacy of opioid agonists (e.g., Adams et al., 1990; Mjanger and Yaksh, 1991; Comer et al., 1992; Zernig et al., 1995a), and the means to make inferences about opioid receptor turnover (e.g., Caruso et al., 1980; Burke et al., 1994; Zernig et al., 1994).

β-FNA is the most thoroughly characterized irreversible opioid antagonist (Takemori et al., 1981; Ward et al., 1982a,b; Takemori and Portoghese, 1985; Mjanger and Yaksh, 1991); β-CNA is less so (Caruso et al., 1980; Ward et al., 1982a; Takemori and Portoghese, 1985). Although β-CNA is thought to bind irreversibly to all opioid binding sites with similar affinity (Ward et al., 1982a), the irreversible antagonist effects of β-FNA are mediated almost entirely via μ-receptors (Takemori et al., 1981; Ward et al., 1982b; Liu-Chen and Phillips, 1987). A drawback to both irreversible antagonists is a reversible κ-opioid activity that initially coincides with μ-opioid effects in vivo, making inferences about whether partial or complete opioid receptors are agonist or antagonist in vivo difficult (Adams et al., 1990; Caruso et al., 1980).

ABBR EVIATI ONS: β-CNA, β-chlornaltrexamine; β-FNA, β-funaltrexamine; C-CAM, clocinnamox; DAMGO, [d-Ala², N-Me-Phe⁴,Gly⁵-ol]-enkephalin; DPDPE, [d-Pen²,d-Pen⁵]-enkephalin; M-CAM, methocinnamox; MM-CAM, 3-methoxymethocinnamox; nor-BNI, norbinaltorphimine; NTI, naltrindole.
with antagonist effects (Takemori et al., 1981; Ward et al., 1982a,b). Because β-FNA and β-CNA are generally used for their antagonist properties, an extended pretreatment time is required in vivo to allow the reversible agonist effect to subside (Ward et al., 1982b; Zimmerman et al., 1987; Broadbear et al., 1994). It can be inferred, however, that both agonist and antagonist activity occur simultaneously, so it is possible that the agonist effects of β-FNA and β-CNA may alter their subsequent antagonist activity. Furthermore, β-FNA alkylates only 50% of μ-opioid receptors (Franklin and Traynor, 1991; Martin et al., 1993). This may be due to differential alkylation of putative subtypes or to different affinity states of the μ-opioid receptor (Tam and Lui-Chen, 1986; Franklin and Traynor, 1991). Also, a dichotomy exists between the recovery of binding and the more rapid recovery of μ-opioid pharmacological effects after β-FNA administration, such as the recovery of heroin self-administration in rats (Martin et al., 1995). Thus, there is a need for additional long-acting μ-opioid antagonists to study these problems.

We have previously reported on the novel, systemically active long-lasting opioid antagonist clonincamox (C-CAM; Comer et al., 1992). In the mouse warm-water tail-withdrawal test, C-CAM suppresses the antinociceptive effect of fentanyl such that the maximal effect is not achieved and withdrawal test, C-CAM suppresses the antinociceptive effect of fentanyl such that the maximal effect is not achieved and was removed in vacuo, and CH2Cl2 (10 ml), 14-aminodihydrocodeinone was collected, dried (Na2SO4), and evaporated to a fawn powder.

This study provides a more detailed characterization of C-CAM and in particular its close analog methocinnamox (M-CAM; 14p-(4'-methylcinnamoylamido)-7,8-dihydro-N-cyclopropylmethyl-normorphine mesylate, Fig. 1) and compares them functionally with β-FNA and β-CNA as tools for the long-lasting blockade of systemically administered μ-opioid agonists. The compounds were evaluated in vivo using the warm-water tail-withdrawal and acetic acid-induced writhing procedures in mice, and the binding of each of the antagonists to opioid receptors was examined in mouse brain using both in vitro and ex vivo methods. The results confirm that C-CAM and M-CAM are devoid of agonist effects both in vitro and in vivo and provide a long-lasting and, in the case of M-CAM, a highly selective blockade of μ-opioid receptors.

Materials and Methods

Animals

Male NIH Swiss mice weighing 20 to 32 g (Harlan Sprague-Dawley, Indianapolis, IN) were housed in standard laboratory cages (8−12/cage) in a temperature-controlled colony room maintained on a 12-h light/dark cycle. Food (Purina Rodent Chow; Purina Mills, St. Louis, MO), and water were available ad libitum until testing. Each animal was used only once and was sacrificed by an overdose of pentobarbital immediately after use. Studies were carried out in accordance with the Declaration of Helsinki and were approved by the National Institutes of Health. The University of Michigan University Committee on the Use and Care of Animals approved the experimental protocols.

Chemicals and Drugs

[1H][α-Ala2,N-Me-Phe4,Gly5-α1]-Enkephalin ([H]DAMGO; 40.7 Ci/mmol) was purchased from Amersham (Arlington Heights, IL). [1H]U69,593 ([H]5α,7α,β(+)-N-methyl-N-[7-(1-pyrrolidinyl)-1-oxaspiro[4,5]dec-8-yl]benzeneacetaamide; 47.9 Ci/mmol) and [1H][α- Pen2,D-Pen5]-enkephalin ([H]DPDPE; 30 Ci/mmol) were obtained from New England Nuclear (Boston, MA). β-CNA was purchased from Research Biochemicals International (Natick, MA). Morphine sulfate was obtained from Mallinckrodt (St. Louis, MO), DAMGO, DPDPE, fentanyl, naloxone, and U69593 were obtained from Sigma Chemical Co. (St. Louis, MO). Naltrindole (NTI) and norbinaltorphine (nor-BNI) were gifts from Dr. B. de Costa (National Institutes of Health, Bethesda, MD). BW373U86 ([H]DPDPE, 30 Ci/mmol) and [3H][D-Ala2, D-Leu5]-enkephalin ([H]DAMGO; 40.7 Ci/mmol) and [3H][D-Ala2, D-Leu5]-enkephalin (10−12 Ci) were purchased from Amersham (Arlington Heights, IL). [3H]U69,593 ([ 3H](5α,7α,β(+)-N-methyl-N-[7-(1-pyrrolidinyl)-1-oxaspiro[4,5]dec-8-yl]benzeneacetaamide; 47.9 Ci/mmol) and [3H][α-Pen2,D-Pen5]-enkephalin ([3H]DPDPE; 30 Ci/mmol) were obtained from New England Nuclear (Boston, MA). β-CNA was purchased from Research Biochemicals International (Natick, MA). Morphine sulfate was obtained from Mallinckrodt (St. Louis, MO). DAMGO, DPDPE, fentanyl, naloxone, and U69593 were obtained from Sigma Chemical Co. (St. Louis, MO). Naltrindole (NTI) and norbinaltorphine (nor-BNI) were gifts from Dr. B. de Costa (National Institutes of Health, Bethesda, MD). β-FNA and C-CAM were synthesized in our laboratories as previously described (Portoghese et al., 1980; Lewis et al., 1988). M-CAM was synthesized by acylation of 14-aminodihydrocodeinone with p-methoxyxycinnamoyl chloride followed by 3-O-demethylation as follows. p-Me-cinnamic acid (201 mg, 1.24 mmol) in anhydrous toluene was refluxed with oxalyl chloride (0.77 ml, 8.8 mmol) for 1 h. The tolucene and excess oxalyl chloride were then removed in vacuo, and CH2Cl2 (10 ml), 14-aminodihydrocodeinone (350 mg, 1.50 mmol), and triethylamine (0.16 ml, 1.1 mmol) were added. Stirring was continued overnight before adding a saturated solution of NaHCO3 and extracting with CH2Cl2. The organic layer was collected, dried (Na2SO4), and evaporated to a fawn powder. Silica gel chromatography (95:4:1, CH2Cl2/MeOH/NH3) yielded 3-methoxymethocinnamox (MM-CAM) as a white solid; 410 mg (84%) calculated for C31H34N2O4.410 mg (84%) calculated for C31H34N2O4.410 mg (84%) calculated for C31H34N2O4.410 mg (84%) calculated for C31H34N2O4.410 mg (84%) calculated for C31H34N2O4.410 mg (84%) calculated for C31H34N2O4.410 mg (84%) calculated for C31H34N2O4.410 mg (84%) calculated for C31H34N2O4.410 mg (84%) calculated for C31H34N2O4.410 mg (84%) calculated for C31H34N2O4.410 mg (84%) calculated for C31H34N2O4.410 mg (84%) calculated for C31H34N2O4.410 mg (84%) calculated for C31H34N2O4.410 mg (84%) calculated for C31H34N2O4.
adding BBr₃ (1 M in CH₂Cl₂, 2.4 ml, 2.4 mmol). The solution was allowed to warm to room temperature over 1 h before pouring into a 1:1 mixture of ice/conc. NH₃. The organic phase was collected, and the aqueous layer was extracted with CH₂Cl₂ (3 × 5 ml). The organic layers were combined and washed with brine (15 ml), dried over Na₂SO₄, and evaporated in vacuo to leave a dark foam. Silica gel chromatography (95:4:1, CH₂Cl₂/MeOH/NH₃) yielded M-CAM as a white solid. 100 mg (52%), δ H (DMSO) 2.33 (3 H, s, Ar-CH₃), 2.95 (1 H, d, J = 18.3 Hz, 1H), 4.88 (1 H, s, 5-H), 6.54 (1 H, d, J = 8.1 Hz, 1-H), 6.59 (1 H, d, J = 8.1 Hz, 2-H), 6.88 (1 H, d, J = 15.6 Hz, COCH=CHAr), 7.24 (2 H, d, J = 8.0 Hz, 3'- and 5'-H), 7.42 (1 H, d, J = 15.6 Hz, COCH=CHAr), 7.52 (2 H, d, J = 8.1 Hz, 2'- and 6'-H); calculated for C₃OH₃₂N₂O₄

**Acetic Acid-Induced Writhing Assay**

The antinociceptive effects and antagonist selectivity of each of the drugs used in this study were assessed using a modification of the acetic acid-induced writhing assay as previously described (Travnor et al., 1999). Mice were injected i.p. with 0.4 ml of 0.6% acetic acid and placed in individual Plexiglas boxes (18 × 28 × 23 cm) for observation. Five minutes later, a 5-min observation period was initiated, during which the number of writhes was recorded. Each writh typically consisted of a wave of contraction of the abdominal musculature followed by extension of the hind legs. Test drug or vehicle was administered either s.c. (agonists) or i.p. (antagonists) at various times before acetic acid administration.

**Agonist Activity.** C-CAM (10 mg/kg) and M-CAM (32 mg/kg) were tested for antinociceptive effects at 15, 30, and 60 min after administration. β-FNA (32 mg/kg) was tested at a number of time points ranging from 10 min to 24 h after administration. The effect of the δ-selective antagonist nor-BNI (32 mg/kg i.p.; 24-h pretreatment) was examined at the time agonist effect was at its maximum. Due to limited availability, single-dose experiments with β-CNA (32 mg/kg) were performed 1 h after administration.

**Antagonist Activity.** The antagonist activity of β-CNA, β-FNA, C-CAM, and M-CAM was tested against morphine (μ-agonist), BW373U86 (δ-agonist), and bremazocine (κ-agonist) using doses and pretreatment times of the antagonists that produced a 5- to 10-fold shift in the morphine dose-effect curve. If no antagonism of either bremazocine or BW373U86 was observed, higher doses of the antagonists were tested.

The number of writhes for each mouse was expressed as a percentage of the control number of writhes per mouse. The control number of writhes per mouse was defined as the mean number of writhes per mouse when s.c. injection of sterile water was administered 15 min before the acetic acid injection. Individual percentage control values for mice in the treatment group were averaged, and the S.E.M. value was calculated. Each data point represents the results from six mice, unless otherwise noted, and each mouse contributed to only one condition in a dose-effect evaluation.

**Warm-Water Tail-Withdrawal Assay**

The antinociceptive effect of morphine in the presence or absence of β-FNA, β-CNA, C-CAM, and M-CAM was measured using the warm-water tail-withdrawal procedure as modified for mice (Burke et al., 1994). Each mouse was restrained in a cylindrical plastic container (Harvard Apparatus, South Natick, MA) that allowed the tail to protrude. The tail was immersed to up to half its length in 55.0 ± 0.2°C water, and the latency to tail withdrawal was measured using a stopwatch. A cumulative dosing procedure for morphine was used whereby testing and injection of the next concentration of morphine took place every 30 min. The initial measurement (baseline latency) was determined 25 min after an injection of sterile water. All injections were given i.p. This injection-testing procedure continued until either the mouse failed to remove its tail after 15 s of immersion (designated as “100% analgesia”) or toxic effects of high doses (e.g., convulsions and/or death) interfered with the measurements.

Data are presented as percentage maximum effect (MPE) = [test latency – baseline latency]/(cutoff time – baseline latency) × 100, where cutoff time was 15 s, and baseline latency is the delay from immersion until withdrawal of the tail from 55°C water after an injection of vehicle (sterile water). The results for mice in a particular group were averaged, and the S.E.M. value was calculated. The dose-effect curves consist of data from at least five mice; each mouse contributed to only one experiment.

**Binding Assays**

**In Vitro**. Homogenates of mouse cerebral cortex were prepared and ligand-binding assays were performed essentially as previously described (Burke et al., 1994). The relative affinities of C-CAM, M-CAM, β-FNA, and β-CNA were determined in competition studies using [3H]DAMGO (0.5 nM) to label δ-sites, [3H]U69,593 (1.5 nM) to label κ-sites, and [3H]DPDPE (1.5 nM) to label δ-sites. Each assay included 0.5 μg homogenate protein (Lowry et al., 1951), radiolabeled ligand, and various concentrations of unlabeled drug in a total volume of 0.525 ml in 50 mM Tris-HCl buffer. Incubations were performed at 25°C for 80 min ([3H]DAMGO), 40 min ([3H]U69,593), or 70 min ([3H]DPDPE). Specific binding was defined in the presence of an excess (10 μM) of the corresponding unlabeled ligand. Reactions were terminated by rapid filtration through glass-fiber disks (Whatman GF/C) treated with water that had been saturated at room temperature with N-aryl alcohol ([3H]DAMGO or [3H]DPDPE assays) or 0.05% polyethyleneimine ([3H]U69,593 assays). Filters were rapidly washed three times with 3 ml ice-cold 50 mM Tris-HCl, pH 7.4, and the radioactivity retained on the filters was determined by liquid scintillation counting.

**Ex Vivo**. Mice were treated s.c. with M-CAM (1.8 mg/kg) or sterile water. Then, 1 h later, the mice were sacrificed, and the brains were rapidly removed. Homogenates of brain (minus cerebellum) were prepared in 10 volumes Tris-HCl (pH 7.4, 50 mM) and centrifuged at 18,000g for 20 min. The resultant pellet was resuspended in Tris-HCl buffer, warmed to 37°C for 20 min to dissociate loosely bound ligands, and then recentrifuged. The final pellet was resuspended in Tris-HCl buffer for binding assays. The specific binding of [3H]DAMGO (1.6 nM), [3H]DPDPE (2.6 nM), and [3H]bremazocine (1 nM in the presence of 1 μM DAMGO and 1 μM DPDPE to block binding to μ- and δ-receptors) was determined at 25°C for 60 min, as earlier, using naloxone (10 μM) to define nonspecific binding.

**[^35S]Guanosine-5'-O-(3-thio)triphosphate (GTPγS) Assays**

C6 glial cells stably transfected with either the rat cloned μ-opioid (C6μ; Alt et al., 1998) or δ-opioid receptor (C6δ; Clark et al., 1997), and CHO cells expressing the human κ-opioid receptor (CHO-κrko; Zhu et al., 1997) were cultured in Dulbecco's modified Eagle's medium (C6 cells) or Dulbecco's modified Eagle's medium/F-12 (CHO-κrko cells), with 10% fetal calf serum and 1.0 to 0.2 mg/ml geneticin. Cells were grown in monolayers to confluency at 37°C in a humidified 5% CO₂ atmosphere. Cells were harvested in HEPES (20 mM, pH 7.4)-buffered saline containing 1 mM EDTA, dispersed by agitation, and collected by centrifugation at 500g. The cell pellet was suspended in a buffer of 20 mM HEPES, pH 7.4, 100 mM NaCl, and 10 mM MgCl₂·6H₂O (buffer A) and homogenized using a tissue tearor (Biospec Products). The resultant homogenate was centrifuged at 50,000g, and the pellet was collected, washed in buffer A, and recentrifuged. The pellet was finally resuspended in buffer A to give a protein concentration of 100 to 200 μg/ml (Lowry et al., 1951) and stored at −80°C. All procedures were performed at 4°C.

Membranes (approximately 50 μg protein) were incubated in buffer A containing [35S]GTPγS (80 pM), GDP (3 μM), and varying
concentrations of test compound in a total volume of 1 ml for 60 min at 30°C as described previously (Traynor and Nahorski, 1995). Non-specific binding was defined using unlabeled GTPgS (10 μM). Bound and free [35S]GTPgS were separated by vacuum filtration through GF/B filters and quantified by liquid scintillation counting. Specific binding was 90 to 95% of total binding. Maximal stimulation of [35S]GTPgS was determined using 10 μM fentanyl (C6m), 10 μM SNC80 (C6d), and 10 μM U69593 (CHO-hkor).

Data Analysis

Behavioral Assays. ED50 values for agonists in the absence and presence of antagonist were calculated as described by Tallarida and Murray (1987). Shifts in ED50 values were determined to be statistically significant if there was no overlap in the 95% confidence intervals.

In Vitro Assays. Data from binding and [35S]GTPgS assays were analyzed by nonlinear regression using Prism version 2.01 (GraphPad Software, San Diego, CA) to give IC50 values for the ligand binding and EC50 values for the [35S]GTPgS assays. IC50 values were converted to Ki values using the Cheng and Prusoff (1973) equation. These data are presented as apparent Ki values due to the likely nonequilibrium properties of the compounds. Data are mean ± S.E. values from at least three experiments performed in triplicate. Statistical significance was determined using Student’s t test.

Results

Antinociceptive Assays

Agonist Activity. In the acetic acid-induced writhing assay, neither C-CAM (10 mg/kg) nor M-CAM (32 mg/kg) produced a significant decrease in writhing relative to the sterile water control at 15-, 30- (data not shown), and 60-min pretreatment times (Fig. 2). In contrast, at 32 mg/kg, both β-CNA and β-FNA were effective in suppressing the writhing response: β-FNA at 15 min after administration and β-CNA at 60 min after administration (Fig. 2). The antinociceptive activity of β-CNA and β-FNA was prevented by pretreatment with the α-selective antagonist nor-BNI administered as a 24-h pretreatment (Fig. 2). None of the compounds, at the doses shown in Fig. 2, showed evidence of agonist activity in the 55°C warm-water tail-withdrawal assay (data not shown).

Antagonist Effects. β-FNA, β-CNA, C-CAM, and M-CAM all antagonized the antinociceptive actions of morphine in the warm-water tail-withdrawal test. At 1 h after the administration of either C-CAM (1 mg/kg; Fig. 3a) or M-CAM (1 mg/kg; Fig. 3b), a rightward shift in the morphine dose-effect curve was observed. A dose-related effect was evident because the higher dose of 3.2 mg/kg caused a more pronounced effect and, in the case of M-CAM, a complete flattening of the morphine dose-response curve (Fig. 3, a and b). β-FNA and

Fig. 2. Effects of C-CAM, M-CAM, β-CNA and β-FNA in the mouse acetic acid-induced writhing assay. Drugs were administered s.c. at the times indicated before assay. The antinociceptive activity of β-CNA and β-FNA was prevented by nor-BNI (32 mg/kg, 24-h pretreatment). Each column represents the mean ± S.E. of the writhing activity of six mice relative to sterile water controls.

Fig. 3. Effects of morphine in the mouse determined in the warm-water tail-withdrawal assay. Morphine-induced antinociception was determined without or after pretreatment at the times indicated with a single doses of C-CAM (a), M-CAM (b), β-CNA (c), and β-FNA 32 (d). A temperature of 55°C was used with a cutoff time of 15 s. Each point represents the mean ± S.E. of the tail withdrawal latency for five mice.
β-CNA also showed a very strong antagonism of the antinociceptive effect of morphine in this assay (Fig. 3, c and d). The order of potency was M-CAM > C-CAM > β-CNA > β-FNA. With all compounds, antagonism was still evident 48 h after administration, but at this time morphine showed recovery to a full antinociceptive effect but with a shift to the right in the dose-effect curve.

The selectivity of C-CAM, M-CAM, β-CNA, and β-FNA for μ-, κ-, and δ-opioid receptors was assessed in the writhing assay against the agonists morphine, bremazocine, and BW373U86, respectively. Morphine dose-dependently suppressed writhing behavior with an ED$_{50}$ of 0.55 mg/kg s.c. (Table 1). C-CAM, M-CAM, β-FNA, and β-CNA produced parallel, dose-dependent rightward shifts in the morphine dose-response curve, although they did so with somewhat different potencies in the order M-CAM >> C-CAM > β-CNA ≥ β-FNA (Table 1). For instance, a dose of 3.2 mg/kg M-CAM produced a 218-fold increase in the ED$_{50}$ for morphine, whereas the same dose of C-CAM resulted in only a 9.5-fold increase. In turn, C-CAM (32 mg/kg) was more potent as an antagonist, causing a 68-fold increase in the ED$_{50}$ for morphine than the same doses of β-CNA (22-fold increase) and β-FNA (11.5-fold increase). However, this difference may relate to the different pretreatment times of the antagonists used. A 1-h pretreatment time was used for C-CAM and M-CAM, but a 24-h pretreatment time was necessary for both β-CNA and β-FNA to avoid the confounding agonist effects of these compounds. A lower dose (3.2 mg/kg) of β-FNA neither showed an agonist effect nor antagonized morphine. As an alternative to overcome the initial, predominant κ-mediated agonist effects of β-FNA, mice were pretreated with nor-BNI (32 mg/kg i.p., 24-h pretreatment). Under these conditions, β-FNA (32 mg/kg; 1-h pretreatment) produced a 35-fold shift in the morphine dose-effect curve, resulting in an ED$_{50}$ for morphine of 19.1 mg/kg (confidence limits 15.1–24.2); that is three times the ED$_{50}$ measured 24 h after the same dose of β-FNA in the absence of nor-BNI pretreatment.

There was no evidence of suppression of the maximal response to morphine in the writhing assay, as shown for M-CAM (Fig. 4). Morphine has agonist actions at κ-receptors in vitro (Toll et al., 1998). To examine whether the response of morphine in the presence of μ-receptor blockade was mediated by κ-receptors, the effect of morphine was reexamined in the presence of M-CAM after a 24-h pretreatment with 32 mg/kg i.p. nor-BNI. This pretreatment with nor-BNI did not significantly shift the dose-effect curve for morphine alone but shifted the ED$_{50}$ for morphine in the presence of M-CAM by 6.9-fold (Fig. 4). This suggested the response to morphine observed in the presence of μ-receptor blockade by M-CAM was mediated through a κ-receptor mechanism.

Antagonism by C-CAM, M-CAM, β-FNA, and β-CNA of κ- and δ-mediated antinociception was also evaluated. Bremazocine, a κ-selective agonist in this assay (Broadbear et al., 1994; ED$_{50}$ = 0.012 mg/kg), was 40 times more potent than morphine in suppressing acetic acid-induced writhing in the mouse, whereas the δ-selective agonist BW373U86 (ED$_{50}$ = 2.8 mg/kg) was 5-fold weaker (Table 1). Pretreatment with 10 mg/kg M-CAM or 32 mg/kg C-CAM produced significant, and approximately equipotent, antagonism of the effects of both agonists (Table 1). However, with both C-CAM and M-CAM, the magnitude of the increases in agonist ED$_{50}$ was smaller for the κ- and δ-agonists than for the μ-agonist morphine. This was especially true for M-CAM, which at a dose of 1.8 mg/kg produced a 74-fold increase in the ED$_{50}$ of morphine but was without significant effect at this dose in blocking bremazocine- or BW373U86-induced antinociception (Table

**TABLE 1**

Potencies of morphine (μ), bremazocine (κ), and BW373U86 (δ) in the mouse acetic acid-induced writhing assay and their antagonism by C-CAM, M-CAM, β-FNA, and β-CNA

<table>
<thead>
<tr>
<th>Antagonist (mg/kg, pretreatment time)</th>
<th>Morphine</th>
<th>Bremazocine</th>
<th>BW373U86</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ED$_{50}$</td>
<td>ED$_{50}$</td>
<td>ED$_{50}$</td>
</tr>
<tr>
<td></td>
<td>Dose Ratio</td>
<td>Dose Ratio</td>
<td>Dose Ratio</td>
</tr>
<tr>
<td>None</td>
<td>0.55 (0.40–0.77)</td>
<td>0.012 (0.01–0.015)</td>
<td>2.81 (1.60–4.93)</td>
</tr>
<tr>
<td>C-CAM</td>
<td>(3.2, 1 h)</td>
<td>(3.2, 1 h)</td>
<td>(3.2, 1 h)</td>
</tr>
<tr>
<td>M-CAM</td>
<td>(32, 1 h)</td>
<td>2.53 (3.16–8.66)</td>
<td>0.03 (0.02–0.05)</td>
</tr>
<tr>
<td></td>
<td>37.2 (19.1–75.6)</td>
<td>0.098 (0.06–0.16)</td>
<td>28.7 (20.5–40.0)</td>
</tr>
<tr>
<td></td>
<td>2.96 (2.00–4.38)</td>
<td>N.T.</td>
<td>10.2</td>
</tr>
<tr>
<td>M-CAM</td>
<td>(1.8, 1 h)</td>
<td>40.8 (19.5–85.5)</td>
<td>0.009 (0.006–0.014)</td>
</tr>
<tr>
<td></td>
<td>110 (66.3–216)</td>
<td>N.T.</td>
<td>2.1 (N.S.)</td>
</tr>
<tr>
<td>β-FNA</td>
<td>(3.2, 1 h)</td>
<td>(3.2, 1 h)</td>
<td>(3.2, 1 h)</td>
</tr>
<tr>
<td></td>
<td>(10.0, 1 h)</td>
<td>N.T.</td>
<td>N.T.</td>
</tr>
<tr>
<td></td>
<td>N.T.</td>
<td>0.085 (0.058–0.13)</td>
<td>15.4 (10.9–21.8)</td>
</tr>
<tr>
<td></td>
<td>6.32 (3.02–13.3)</td>
<td>7.1</td>
<td>5.5</td>
</tr>
<tr>
<td></td>
<td>21.4 (17.4–26.4)</td>
<td>2.0</td>
<td>2.46 (1.63–3.74)</td>
</tr>
<tr>
<td>β-CNA</td>
<td>(32, 24 h)</td>
<td>11.8 (7.54–18.5)</td>
<td>0.25 (0.07–0.89)</td>
</tr>
<tr>
<td></td>
<td>21.5</td>
<td>20.8</td>
<td></td>
</tr>
</tbody>
</table>

N.S., not significantly different from vehicle-pretreated animals; N.T., not tested.
1). In contrast, β-FNA was ineffective at antagonizing the δ-selective agonist, even at a dose of 32 mg/kg, and at this dose shifted only the dose-effect curve for bremazocine by 2-fold, clearly showing a preference for antagonism of morphine-mediated antinociception. β-CNA produced similar shifts in the dose-effect curves of morphine, bremazocine, and BW373U86 (Table 1).

**Ligand-Binding Assays**

In mouse brain homogenates, β-FNA and β-CNA as well as C-CAM and M-CAM had nanomolar affinity for all three opioid receptors, with limited selectivity for the μ-receptor. In the case of C-CAM and M-CAM, receptor preference was in the order μ > δ > κ, although the differences were not very pronounced. In contrast, β-FNA and β-CNA had similar affinity for μ- and κ-receptors but much reduced affinity for the δ-receptor (Table 2).

To study opioid binding ex vivo, mice were injected with M-CAM (1.8 mg/kg s.c.) 1 h before the removal of brains and preparation of homogenates. At radioligand concentrations approximating their affinities, the specific binding of [3H]DAMGO was reduced by 76% in washed brain homogenates from M-CAM-treated animals compared with saline-treated controls, but the binding of [3H]DPDPE and [3H]U69593 was unchanged (Fig. 5). In experiments using nonwashed, crude homogenates in which the mice brains were simply homogenized in 100 ml Tris-HCl buffer/75 mg brain tissue, the binding of [3H]DAMGO was reduced by 72.0 ± 17.5%, [3H]DPDPE was reduced by 25.0 ± 5.1%, and [3H]bremazocine (in the presence of μ- and δ-receptor blockade) was reduced by 14.6 ± 2.6% compared with saline-treated controls.

**[35S]GTPγS Assays**

At a concentration as high as 1 μM, M-CAM caused only a 7.2 ± 1.9% (n = 3) stimulation of [35S]GTPγS binding to κ-opioid receptors expressed in CHO cells compared with the full agonist U69593 and a 12.7 ± 1.9% (n = 5) stimulation of μ-opioid receptors in C6 cells compared with the full μ-agonist fentanyl. These very low levels of stimulation of [35S]GTPγS binding, at concentrations many times greater than the binding affinity of the compounds, do not lead to agonist effects in vitro or in vivo preparations (Traynor et al., 1999). At δ-opioid receptors expressed in C6 cells, M-CAM did not stimulate [35S]GTPγS binding.

**Discussion**

The aim of this work was to determine whether cinnamoylamidomorphinans such as C-CAM and M-CAM are functionally superior to β-FNA and β-CNA for blocking the μ-opioid receptor population, thereby allowing study of the physiological and pharmacological roles of this receptor.

The order of potency of the long-lasting antagonists in preventing morphine antinociception in the writhing assay and the tail-withdrawal assay was M-CAM > C-CAM > β-CNA > β-FNA. The warm-water tail-withdrawal assay cannot be used to accurately assess antagonist potency because these agents caused a flattening of the morphine dose-effect curve, nor can the assay be used to test ligand selectivity because systemically administered κ- and δ-selective agonists generally have no antinociceptive efficacy in thermal assays (Hayes et al., 1985; Mjanger and Yaksh, 1991). In the writhing assay, however, both selectivity and antagonist potency can be determined. In the writhing assay, β-FNA showed a selective antagonism of the μ-receptor as expected (Takemori et al., 1981; Ward et al., 1988b). This contrasted with its binding profile, which indicated a lack of selectivity between μ- and κ-receptors. However, this is not important in governing its antagonist selectivity in vivo because interaction with the κ-receptor results in agonism and is reversible. In contrast, β-CNA was nonselective, producing comparable shifts in the dose-effect curves of morphine, bremazocine, and BW373U86, in agreement with its profile in binding assays.

C-CAM and M-CAM showed preference for μ-antagonism and were weaker but equipotent as κ- and δ-antagonists. M-CAM in particular showed a striking selectivity for μ-receptors. The selectivity and antagonist potency difference between M-CAM and C-CAM were not expected due to the very close similarity in the structure of these compounds. Nevertheless, compounds in this series do show very marked pharmacological variations with only small structural changes (Nieland et al., 1995), suggesting very subtle interations of the cinnamoylamido substituent with the receptor binding domain. The finding of a marked in vivo selectivity of M-CAM also contrasts with the in vitro binding data in which only a 3.7-fold selectivity of M-CAM for μ- over δ-receptors and an 8-fold selectivity for μ- over κ-receptors were evident. This difference is perplexing, but there is evidence that different measures of antagonist affinity do not always coincide with effects seen in pharmacological assays, especially when binding assays are performed in low ionic strength Tris-HCl buffer rather than in physiological medium. For example, the μ-antagonist cyprodime shows very different affinities for κ-

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**TABLE 2**

Affinities ([Kᵦ]) of C-CAM, M-CAM, β-FNA, and β-CNA for μ-, δ-, and κ-receptors in mouse brain homogenates

<table>
<thead>
<tr>
<th></th>
<th>μ</th>
<th>κ</th>
<th>δ</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-CAM</td>
<td>0.7 ± 0.1</td>
<td>5.7 ± 0.9</td>
<td>1.9 ± 0.2</td>
</tr>
<tr>
<td>M-CAM</td>
<td>0.6 ± 0.1</td>
<td>4.9 ± 0.6</td>
<td>2.2 ± 0.3</td>
</tr>
<tr>
<td>β-FNA</td>
<td>1.1 ± 0.1</td>
<td>3.4 ± 0.5</td>
<td>78.7 ± 2.3</td>
</tr>
<tr>
<td>β-CNA</td>
<td>2.0 ± 0.3</td>
<td>2.5 ± 0.1</td>
<td>63.6 ± 1.3</td>
</tr>
</tbody>
</table>

Values were obtained from displacement curves against 0.5 nM [3H]DAMGO (μ, 1.5 nM [3H]U69593 (κ), and 1.5 nM [3H]DPDPE (δ) as described under Materials and Methods. Values represent the means ± S.E. of at least three experiments.
and δ-receptors in binding assays than are apparent from pharmacological measures of its affinity (Schmidhammer et al., 1995). Furthermore, the pseudoirreversible nature of the binding that occurs in vivo provides a second recognition step that is likely to enhance selectivity (Takemori and Portoghese, 1985). Importantly, the in vivo findings were confirmed by ex vivo binding showing that the majority of μ-opioid binding of [3H]DAMGO was lost after the pretreatment of mice for 1 h with M-CAM, whereas no loss of δ- or κ-binding was seen. In nonwashed membranes, there was some inhibition of δ- and κ-binding, suggesting a reversible interaction with these receptors. The cinnamoylamidomorphinans do not alkylate the μ-receptor (Zernig et al., 1995b; McLaughlin et al., 1999), so these results confirm that very high-affinity, pseudoirreversible binding develops rapidly in vivo at the μ-receptor (Piggot et al., 1995) but not at δ- or κ-receptors. This is consistent with the resistance of C-CAM binding to extensive washing (Zernig et al., 1996).

The large, insurmountable shift caused by the antagonists in the morphine dose-effect curve in the warm-water tail-withdrawal assay and their long duration of action are further evidence that their interaction with the μ-opioid receptor occurs through a nonequilibrium mechanism (Kenakin, 1997). In contrast, in the writhing assay, antagonism was surmountable. The inability of a nonequilibrium antagonist to depress agonist dose-effect curves under some experimental conditions has been reported previously using β-FNA (Takemori et al., 1981; Ward et al., 1982b). Morphine is 30 times more potent in the writhing assay than in the tail-withdrawal assay, implying that fewer receptors have to be occupied to reach full antinociception in the former assay, so there is a greater μ-receptor reserve. Thus, with a reduction in the total number of available μ-receptors, there may still be a sufficient number remaining for a maximal response to be reached. However, it is also feasible that with a large fraction of the μ-receptor population blocked by antagonist, morphine can act through non-μ-opioid receptors. The present results show that when μ-receptors are depleted with M-CAM, morphine exerts its antinociceptive action through κ-receptors. A study in μ-receptor-deficient transgenic mice has suggested that the antinociceptive effect of low doses of morphine (up to 6 mg/kg) is mediated only through μ-receptors (Matthes et al., 1996). That study used heat as the nociceptive source in the hot-plate and tail-withdrawal assays. As discussed earlier, these are likely to be μ-selective assays, so any κ-effects of morphine would not be apparent.

In support of the present finding, morphine is known to act through the κ-receptor in vitro in the presence of β-FNA (Ward et al., 1982a; Franklin and Traynor, 1991), and Take-mori and Portoghese (1987) showed that the agonist effects of morphine in β-FNA-treated mice are mediated by κ-opioid receptors in the mouse writhing assay.

Agonist activity in a compound that is used for its antagonist properties may be a complicating factor. No agonist activity was evident even with high doses of M-CAM or C-CAM, a finding confirmed for M-CAM in the [35S]GTPγS assay at cloned opioid receptors. On the other hand, β-CNA and β-FNA were fully effective antinociceptive agents in the mouse writhing assay through a nor-BNI-sensitive κ-receptor mechanism, as reported previously (Takemori et al., 1981; Ward et al., 1982b), and in CHO-hkor cells, β-FNA shows partial κ-agonist activity (Zhu et al., 1997; Toll et al., 1998).

Antinociception with β-FNA and β-CNA in the writhing assay was observed at doses that produced potent antagonism of morphine at later time points. Furthermore, it is clear that both the agonist and antagonist effects of β-CNA and β-FNA occurred simultaneously as both compounds potently antagonized morphine 1 h after administration in the tail-withdrawal assay, a time at which they had pronounced agonist effects in the writhing assay. Because of such agonist effects, these compounds are generally given as a 24-h pretreatment (Zimmerman et al., 1987; Mjanger and Yaksh, 1991; Ward et al., 1992b; Martin et al., 1995).

Bidlack and colleagues have reported on compounds related to C-CAM and M-CAM that contain the 14β-(p-nitrocinnamoylamido) group, including the metapen derivative 5β-methyl-14β-(p-nitrocinnamoylamo)ino)-7,8-dihydromorphi-

none and its N-cyclopropylmethyl counterpart N-cyclopropyl-

methylnor-5β-methyl-14β-(p-nitrocinnamoylamo)ino)-7,8-
dihydromorphinone (Jiang et al., 1994; McLaughlin et al., 1999). The compounds, particularly the N-methyl derivative, are selective for the μ-opioid receptor. They show no agonist activity when given i.c.v. to the mouse using the tail-flick assay but do show long-lasting antagonism. The lack of agonism is surprising given the agonist efficacy of metapon, but unfortunately, the compounds have not been studied in the writhing assay, which requires much less efficacy in a compound for the manifestation of antinociception. However, taken together, these findings suggest that this class of substituted 14-aminomorphinones is devoid of agonist properties and so represents superior long-lasting μ-opioid receptor antagonists.

In summary, M-CAM and, to a lesser extent, C-CAM have antagonist activity selectively at the μ-opioid receptor and show no evidence of any agonist activity in nociceptive tests. In contrast, both β-CNA and β-FNA have potent antinociceptive activity in the writhing assay at doses and pretreatment times that coincide with those that are optimal for utilization of their nonequilibrium antagonist properties. M-CAM and C-CAM are nonselective in binding assays, suggesting an interaction in vivo that results in the formation of a high-affinity, nonwashable interaction selectively with the μ-receptor. The superior selectivity of M-CAM suggests this to be the nonequilibrium antagonist of choice for the long-term blockade of μ-opioid receptors in vivo.

**References**


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