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14β-Chlorocinnamoylamino derivatives of metopon: long-term μ-opioid receptor antagonists

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Abstract

The affinity, selectivity and antinociceptive properties of 5β-methyl-14β-(*p*-chlorocinnamoylamino)-7,8-dihydromorphinone (MET-Cl-CAMO) and *N*-cyclopropyl-methyl-5β-methyl-14β-(*p*-chlorocinnamoylamino)-7,8-dihydronormorphinone (*N*-CPM-MET-Cl-CAMO) for the multiple opioid receptors were characterized. In competition binding assays using bovine striatal membranes, both compounds inhibited the binding of 0.25 nM [^{3}H][D-Ala^2,(Me)-Phe^4,Gly(ol)^5]enkephalin (DAMGO) with IC_{50} values of less than 2 nM. Preincubation of membranes with MET-Cl-CAMO and *N*-CPM-MET-Cl-CAMO produced a concentration-dependent, wash-resistant inhibition of μ-opioid receptor binding. Saturation binding experiments with *N*-CPM-MET-Cl-CAMO showed a reduction in the number of μ-opioid binding sites without a change in affinity. In the mouse 55°C warm-water tail-flick assay, neither MET-Cl-CAMO nor *N*-CPM-MET-Cl-CAMO at doses up to 100 nmol produced antinociception after intracerebroventricular administration, but morphine-induced antinociception was antagonized in a time- and dose-dependent manner by both compounds. The antagonism produced by 1 nmol of either MET-Cl-CAMO or *N*-CPM-MET-Cl-CAMO reached a maximal effect after 24 h, and lasted up to 48 h. Analgesia mediated by δ- or κ-opioids was not altered by either compound. In summary, the data suggest that MET-Cl-CAMO and *N*-CPM-MET-Cl-CAMO are long-term, μ-opioid receptor antagonists, devoid of agonist properties in the mouse tail-flick assay, and that *N*-CPM-MET-Cl-CAMO may produce its antagonistic effects by binding irreversibly to the μ-opioid receptor.

Keywords: Morphine derivative; Cinnamoylamino group; β-Endorphin receptor; (Irreversible antagonist); Analgesia; (Mouse)

1. Introduction

Irreversible opioid ligands are valuable tools for the characterization of opioid receptors, both in vivo and in vitro (Portoghese et al., 1980; Rice et al., 1983; Bidlack et al., 1990). Addition of site-directed alkylating groups to dihydromorphine, dihydromorphinone, naltrexone and naltrexamine has yielded increased selectivity and affinity of irreversible opioid antagonists (Pasternak and Hahn, 1980; Jiang et al., 1992; Derrick et al., 1996). β-Funaltrexamine, with the structure of naltrexone but containing a fumaramate group in the C-6 position, is one such compound (Portoghese et al., 1980). The fumaramate side chain is an α,β-unsaturated carbonyl, and is thought to act as a Michael acceptor for nucleophilic attack, providing long-term antagonism by forming irreversible covalent bonds with the μ-opioid receptor (Ward et al., 1982, 1985; Liu-Chen and Phillips, 1987). However, use of the selective μ-opioid receptor antagonistic properties of β-funaltrexamine is complicated, requiring a 24 h delay between administration and use to avoid κ-opioid receptor-mediated agonism (Ward et al., 1982). This has led to further research for compounds that act as irreversible opioid receptor antagonists, but with greater ease of use. Under alkaline conditions, [^{3}H]14β-(bromoacetamido)-7,8-dihydromorphine (H_2BAM), a dihydromorphine derivative with a bromoacetamido group in the C-14β position, selectively labeled a μ-opioid binding site in bovine striatal membranes through formation of a covalent bond (Bidlack et al., 1993). However, the alkaline conditions preclude its use in vivo. 14β-(*p*-Chlorocinnamoylamino)-7,8-dihydro-*N*-(cyclopropylmethyl)-normorphinone (C-CAM) is a derivative of naltrexone with a chlorocinnamoylamino group at the C-14 position, replac-

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ing the 14β-hydroxyl group (Lewis et al., 1988). C-CAM was characterized as a long-term μ-opioid receptor antagonist in the mouse warm-water tail-flick assay (Aceto et al., 1989; Comer et al., 1992). After peripheral administration, C-CAM shifted the analgesic dose-response curve of morphine rightward and downward, suggesting C-CAM may act through a nonequilibrium mechanism at opioid receptors to produce its antagonistic activity (Comer et al., 1992; Burke et al., 1994). Recently, seeking further increases in μ-opioid receptor affinity, cinnamoylamino side chains were added at the 14β position of metopon, 5β-methyl-7,8-dihydromorphinone (Sebastian et al., 1993; McLaughlin et al., 1995). Two derivatives containing 14β-*p*-nitrocinnamoylamino constituents, 5β-methyl-14β-(*p*-nitrocinnamoylamino)-7,8-dihydromorphinone (MET-CAMO) and its corresponding *N*-cyclopropylmethyl analog, *N*-CPM-MET-CAMO, demonstrated high affinity and selectivity for the μ-opioid receptor in competition binding experiments with bovine striatal membranes, and antagonized morphine-induced antinociception for up to 72 h in the mouse 55°C warm-water tail-flick assay (Jiang et al., 1993a, 1994). MET-CAMO and *N*-CPM-MET-CAMO did not antagonize antinociception induced by either δ- or κ-opioid receptor selective agonists. An intracerebroventricular (i.c.v.) injection of either compound into mice shifted the dose-response line for morphine-induced antinociception rightward and downward, suggesting an irreversible covalent interaction of the compounds with the μ-opioid receptor (Jiang et al., 1994). The cinnamoylamino group in all three affinity ligands may act as a Michael acceptor, similar to the fumaramate group in β-funaltrexamine, allowing nucleophilic addition with either primary amino groups or thiol groups in the opioid receptor to confer long-term antagonist effect. Recently, β-funaltrexamine has been shown to form a covalent bond with lysine 233 in the cloned rat μ-opioid receptor (Chen et al., 1996). Evidence from pharmacological and receptor sequencing studies suggests the opioid receptors possess an essential sulfhydryl group at or near the opioid binding site (Simon et al., 1973; Pasternak et al., 1975; Childers, 1984; Thompson et al., 1993; Joseph and Bidlack, 1994). However, the cinnamoylamino constituent has not been directly observed to react with thiol groups; when incubated with *N*-acetylcysteine, *N*-CPM-MET-CAMO was recovered unchanged (Sebastian et al., 1993). Moreover, recent experiments incubating mouse brain membranes with [^{3}H]C-CAM, followed by acid precipitation of the protein, did not detect specific radiolabelling of receptor protein (Zernig et al., 1995), suggesting [^{3}H]C-CAM did not bind covalently to the μ-opioid receptor. However, preliminary studies demonstrated that [^{3}H]14β-(*p*-nitrocinnamoylamino)-7,8-dihydro-*N*-cyclopropylmethyl-norcodeinone ([^{3}H]*N*-CPM-CACO) bound covalently to the μ-opioid receptor in bovine striatal membranes (McLaughlin et al., in press). Given these conflicting data, it remains unclear whether 14β-cinnamoylamino derivatives of dihydromorphinone bind covalently to the μ-opioid receptor. Furthermore, it is unclear whether the nitro- or the chlorocinnamoylamino group is more effective in producing long-term opioid receptor antagonism, as all comparisons to date have been between opioid ligands with structural differences aside from the cinnamoyl groups. Direct comparison of the two cinnamoyl groups would require synthesis of both nitro- and chlorocinnamoyl derivatives of the same opioid affinity compound. Finally, given the difficulties in using the selective μ-opioid receptor antagonistic properties of β-funaltrexamine, new irreversible opioid receptor antagonists may prove useful in research if they possess greater efficacy or selectivity for the opioid receptor.

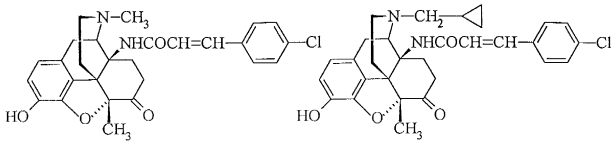

Fig. 1. Structures of MET-Cl-CAMO and its *N*-cyclopropylmethyl analog, *N*-CPM-MET-Cl-CAMO.

The present study characterized two derivatives of metopon, 5β-methyl-14β-(*p*-chlorocinnamoylamino)-7,8-dihydromorphinone (MET-Cl-CAMO) and *N*-cyclopropylmethyl-5β-methyl-14β-(*p*-chlorocinnamoylamino)-7,8-dihydronormorphinone (*N*-CPM-MET-Cl-CAMO) (Fig. 1), for irreversible properties in receptor binding assays and in the mouse 55°C warm-water tail-flick test as a measure of antinociception. Like MET-CAMO and *N*-CPM-MET-CAMO, both derivatives are 5-methyl-7,8-dihydromorphinones but, like C-CAM, both possess a chlorocinnamoylamino group instead of the nitrocinnamoylamino group in MET-CAMO and *N*-CPM-MET-CAMO in the C-14β position, allowing for direct comparison in opioid receptor antagonist efficacy between the nitro- and chlorocinnamoylamino groups. A preliminary study suggested both compounds had highest affinity for the μ-opioid receptor, and produced wash-resistant inhibition of μ-opioid receptor binding (McLaughlin et al., 1994).

2. Materials and methods

2.1. Synthesis of MET-Cl-CAMO and N-CPM-MET-Cl-CAMO

2.1.1. Preparation of MET-Cl-CAMO

To a solution of 150 mg of 14β-amino-5β-methyl-7,8-dihydrocodeinone in 20 ml of methylene chloride and 64 μl of triethylamine there was added dropwise with stirring a solution of 64 μl of *p*-chlorocinnamoyl chloride in 5 ml of methylene chloride in an atmosphere of dry nitrogen. After 3 h, there was added a sodium bicarbonate solution.

The layers were separated and the organic layer washed with H_2O and dried. The solvent was evaporated and the residue was chromatographed on a preparative plate using methylene chloride/methanol (94:6) as the developing solvent. There was obtained 160 mg of the product, 5β-methyl-14β-(*p*-chlorocinnamoylamino)-7,8-dihydrocodeinone MET-Cl-CACO, the codeinone precursor of MET-Cl-CAMO.

100 mg of this product was demethylated with the aid of 97 μl of boron tribromide in 5 ml of dry methylene chloride at −20°C. The mixture was allowed to warm to room temperature and after 1.5 h the reaction mixture was treated with sodium bicarbonate solution. The separated organic layer was washed with water, dried and evaporated to dryness. After purification by thin-layer chromatography (chloroform/methanol, 9:1), 35 mg of MET-Cl-CAMO (Fig. 1) was obtained, with a melting point of 180–186°C (dec). ^{1}H nuclear magnetic resonance analysis also confirmed the structure of the compound.

2.1.2. Preparation of N-CPM-MET-Cl-CAMO

70 mg of *p*-chlorocinnamic acid was added to 3 ml of thionyl chloride and the mixture was refluxed for 1 h. The thionyl chloride was removed in vacuo and the residue, the desired acid chloride, was dissolved in 3 ml of dry methylene chloride and added dropwise with stirring to 90 mg of 14β-amino-5β-methyl-7,8-dihydrocodeinone and 35 μl of triethylamine dissolved in 30 ml of methylene chloride kept at −10°C under an atmosphere of N_2. The reaction mixture was stirred at room temperature for 1 h, then worked up as described above.

The crude product was not purified but was dissolved in 8 ml of dry methylene chloride, cooled to −78°C and treated with 100 ml of boron tribromide. The mixture was stirred for 2 h as the temperature rose to −10°C. After stirring for another hour at room temperature, the mixture was worked up as in the case of MET-Cl-CAMO to give 60 mg of *N*-CPM-MET-Cl-CAMO (Fig. 1), with a melting point of 160–170°C (dec).

2.2. Opioid binding to bovine striatal membranes

Bovine striatal membranes were prepared and competition binding experiments were performed with MET-Cl-CAMO and *N*-CPM-MET-Cl-CAMO to determine affinity of the compounds for the multiple opioid receptors as previously described (Jiang et al., 1994). Briefly, bovine striatal membranes were incubated with 12 different concentrations of the competitor and the radiolabeled ligands at 25°C in a final volume of 1 ml of 50 mM Tris-HCl, pH 7.5. Incubation times of 60 min were used for the μ-opioid receptor selective peptide [^{3}H][D-Ala^2,(Me)-Phe^4,Gly(ol)^5]enkephalin (DAMGO) and the κ-opioid receptor selective ligand [^{3}H](5α,7α,8β)-*N*-methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro(4,5)dec-8-yl]-benzeneacetamide (U69,593), and a 4-h incubation was used to insure equilibrium binding with the δ-opioid receptor selective peptide [^{3}H][D-Pen^2, *p*-Cl-Phe^4,D-Pen^5]enkephalin (pCl-DPDPE) in the presence of 5 mM $MgCl_2$ and 0.1 mM phenylmethylsulfonyl fluoride. To determine the IC_{50} values for the inhibition of binding by the compounds, the final concentrations of [^{3}H]DAMGO, [^{3}H]pCl-DPDPE and [^{3}H]U69,593 were 0.25 nM, 0.2 nM and 1 nM, respectively. Nonspecific binding was measured with inclusion of 10 μM naloxone. Binding was terminated by filtering the samples through Schleicher & Schuell No. 32 glass fiber filters using a Brandel 48-well cell harvester. Filters were soaked at least 60 min in 0.25% polyethylenimine for [^{3}H]pCl-DPDPE and [^{3}H]U69,593 binding experiments. After filtration, filters were washed three times with 3 ml of cold 50 mM Tris-HCl, pH 7.5 and were counted in 2 ml of Ecoscint A scintillation fluid.

To determine the concentration of MET-Cl-CAMO and *N*-CPM-MET-Cl-CAMO required to obtain wash-resistant inhibition of opioid binding, 10 mg of membrane protein were incubated at 37°C for 15 min with seven concentrations of each compound, ranging from 1.6 to 1600 nM, in a final volume of 2 ml of 50 mM Tris-HCl, pH 7.5. An incubation time of 15 min was chosen after demonstration that the corresponding nitrocinnamoylamino compounds did not increase wash-resistant inhibition of opioid binding significantly after this point in time course binding experiments (Jiang et al., 1994). The contents of the tubes were then diluted to 40 ml with cold Tris buffer and centrifuged at $39\,000 \times g$ for 15 min at 4°C. The washing step was repeated for a total of four times. Finally, the membranes were resuspended in 2 ml of 50 mM Tris-HCl, pH 7.5, and opioid binding to 0.2 ml of membranes was determined as described above.

To determine whether the affinity ligands altered the affinity or reduced the number of opioid binding sites, 10 mg of membranes were incubated with 100 nM MET-Cl-CAMO for 15 min, with a final volume of 2 ml of 50 mM Tris-HCl, pH 7.5, followed by four centrifugal washes. *N*-CPM-MET-Cl-CAMO at final concentrations of 8 nM (for the study of μ-opioid receptor binding) and 100 nM (for the study of κ-opioid receptor binding) were incubated with membranes in parallel studies. [^{3}H]DAMGO binding at concentrations ranging from 0.025 nM to 12.8 nM, and [^{3}H]U69,593 binding at concentrations ranging from 0.025 nM to 6.4 nM, was measured as described above. The protein concentration of membranes was determined by the method of Bradford (1976), using bovine serum albumin as the standard.

2.3. Animals

All experiments used male, ICR mice (24–30 g, Harlan Sprague Dawley (Indianapolis, IN, USA)). Mice were kept in groups of eight in a temperature controlled room with a 12-h light-dark cycle. Food and water were available ad libitum until the time of the experiment.

2.4. Injection techniques

Intracerebroventricular injections were made directly into the lateral ventricle according to the modified method of Haley and McCormick (1957). The volume of all i.c.v. injections was 5 μl, using a 10-μl Hamilton microliter syringe. The mouse was lightly anesthetized with ether, an incision was made in the scalp, and the injection was made 2 mm lateral and 2 mm caudal to bregma at a depth of 3 mm.

2.5. Tail-flick assay

The thermal nociceptive stimulus was 55°C water, with the latency to tail flick or withdrawal taken as the endpoint (Vaught and Takemori, 1979). After determining control latencies, the mice received graded i.c.v. doses of opioid agonists or antagonists. Morphine sulfate, DPDPE and U50,488 were each given as single i.c.v. injections with antinociceptive effect measured 20 min after injection unless otherwise stated. In the antagonistic study, various doses of either MET-Cl-CAMO or *N*-CPM-MET-Cl-CAMO were given as a single pretreatment at 20 min, 4, 8, 16, 24, 48, 72 and 96 h before testing. A cut-off time of 15 s was used; if the mouse failed to display a tail flick in that time, the tail was removed from the water and the animal assigned a maximal antinociceptive score of 100%. Mice which showed no response within 5 s in the initial control test were eliminated from the experiment. At each time point, antinociception was calculated according to the following formula: % antinociception = 100 × (test latency − control latency)/(15 − control latency).

2.6. Materials

[^{3}H]DAMGO (60 Ci/mmol) and [^{3}H]U69,593 (64 Ci/mmol) were purchased from Amersham (Arlington Heights, IL, USA). [^{3}H]pCl-DPDPE (48.6 Ci/mmol) was obtained from New England Nuclear (Boston, MA, USA). Morphine sulfate was purchased from Mallinckrodt (St. Louis, MO, USA). DPDPE was purchased from Bachem (Torrance, CA, USA). U50,488 was obtained from Research Biochemicals International (Natick, MA, USA). Ecoscint A was obtained from National Diagnostics (Atlanta, GA, USA). Schleicher & Schuell No. 32 glass fiber filters were obtained from Schleicher & Schuell (Keene, NH, USA).

For radioligand binding assays, MET-Cl-CAMO and *N*-CPM-MET-Cl-CAMO were dissolved in dimethyl sulfoxide at a concentration of 10^{-3} M. Subsequent dilutions were made into 50 mM Tris-HCl, pH 7.5. In the mouse tail-flick assays, MET-Cl-CAMO and *N*-CPM-MET-Cl-CAMO were dissolved in 20% dimethyl sulfoxide, a concentration that did not produce any detectable effect.

2.7. Statistics

IC_{50} values were calculated by least squares fit to a logarithm-probit analysis. The K_i values of unlabelled compounds were calculated from the equation $K_i = IC_{50}/(1 + S)$, where S = (concentration of radioligand)/(K_D of radioligand) (Cheng and Prusoff, 1973). Saturation [^{3}H]DAMGO and [^{3}H]U69,593 binding data were analyzed by nonlinear regression analysis using the LIGAND program (Munson and Rodbard, 1980). Regression lines, D_{50} values, the dose producing 50% antinociception, and 95% confidence limits were determined with each individual data point using the computer program by Tallarida and Murray (1986). All data points shown are the mean of 7–10 mice, with S.E.M. represented by error bars.

3. Results

3.1. Affinity and selectivity of MET-Cl-CAMO and N-CPM-MET-Cl-CAMO for the multiple opioid receptors in bovine striatal membranes

Both MET-Cl-CAMO and *N*-CPM-MET-Cl-CAMO (Fig. 1) demonstrated higher affinity for the μ-opioid receptor than for the δ- or κ-opioid receptors, as determined by their IC_{50} values for the inhibition of μ-, δ- and

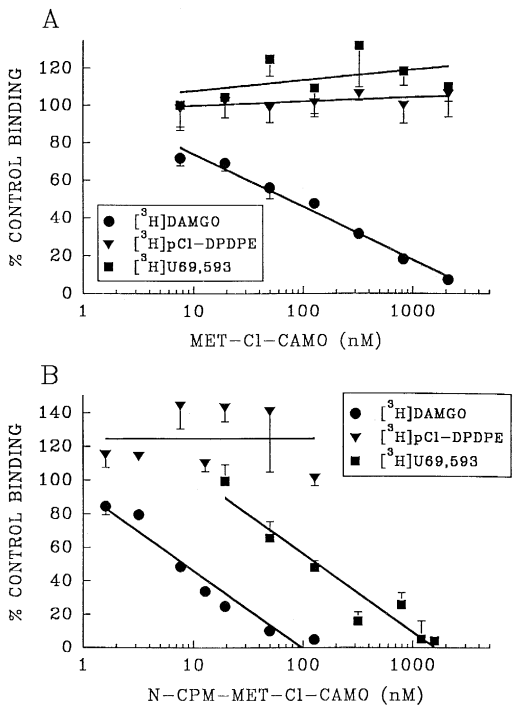

Fig. 2. Wash-resistant inhibition of opioid binding produced by preincubation of bovine striatal membranes with MET-Cl-CAMO and *N*-CPM-MET-Cl-CAMO. Membrane protein at a concentration of 10 mg was incubated with varying concentrations of MET-Cl-CAMO (A) and *N*-CPM-MET-Cl-CAMO (B) at 37°C for 15 min, followed by four centrifugal washes. Binding of 0.25 nM [^{3}H]DAMGO, 0.2 nM [^{3}H]pCl-DPDPE and 1 nM [^{3}H]U69,593 to 0.2 ml of membranes was measured as described in Section 2. Data are presented as the mean percentage of control binding ± S.E.M. from three or more experiments performed in triplicate.

Table 1
IC_{50} values for the inhibition of μ-, δ-, and κ-opioid binding to bovine striatal membranes by MET-Cl-CAMO and *N*-CPM-MET-Cl-CAMO

Radiolabeled ligands	IC_{50} (nM) ± S.E.M.	
	MET-Cl-CAMO	*N*-CPM-MET-Cl-CAMO
[^{3}H]DAMGO (μ)	0.44 ± 0.17	0.91 ± 0.33
[^{3}H]pCl-DPDPE (δ)	48.6 ± 18.0	23.2 ± 2.44
[^{3}H]U69,593 (κ)	13.2 ± 0.42	11.6 ± 3.04

Bovine striatal membranes were incubated with 12 different concentrations of MET-Cl-CAMO and *N*-CPM-MET-Cl-CAMO in the presence of either 0.25 nM [^{3}H]DAMGO, 0.2 nM [^{3}H]pCl-DPDPE or 1 nM [^{3}H]U69,593 in 50 mM Tris-HCl, pH 7.5, at 25°C. After equilibrium binding was reached, membranes were filtered onto glass-fiber filters. Data are listed as the mean IC_{50} values ± S.E.M. from three or more experiments, performed in triplicate.

κ-opioid binding to bovine striatal membranes, measured with [^{3}H]DAMGO, [^{3}H]pCl-DPDPE and [^{3}H]U69,593, respectively (Table 1). MET-Cl-CAMO displayed 2-fold higher affinity for the μ-opioid receptor than *N*-CPM-MET-Cl-CAMO in the competition binding assays, but

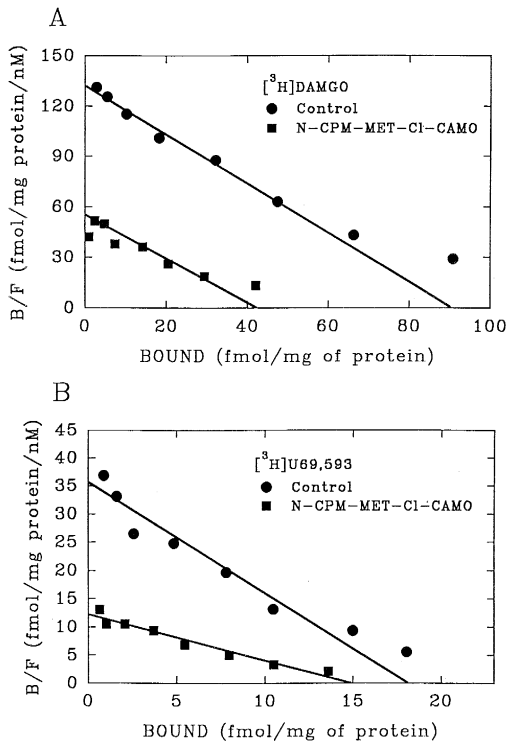

Fig. 3. Scatchard representation of saturation binding to control and *N*-CPM-MET-Cl-CAMO-treated membranes with the receptor-selective opioids [^{3}H]DAMGO (A) and [^{3}H]U69,593 (B). Bovine striatal membranes were incubated in the presence or absence of either 8 or 100 nM *N*-CPM-MET-Cl-CAMO for [^{3}H]DAMGO and [^{3}H]U69,593 experiments, respectively, for 15 min at 37°C. Control samples lacked compound in the preincubation. Membranes were then diluted and submitted to four centrifugal washes. Binding was then measured as described in Section 2, with concentrations of [^{3}H]DAMGO (A) ranging from 0.025 to 3.2 nM, and concentrations of [^{3}H]U69,593 (B) ranging from 0.05 to 6.4 nM. Data are from a representative experiment, which was repeated three times. B/F, bound/free.

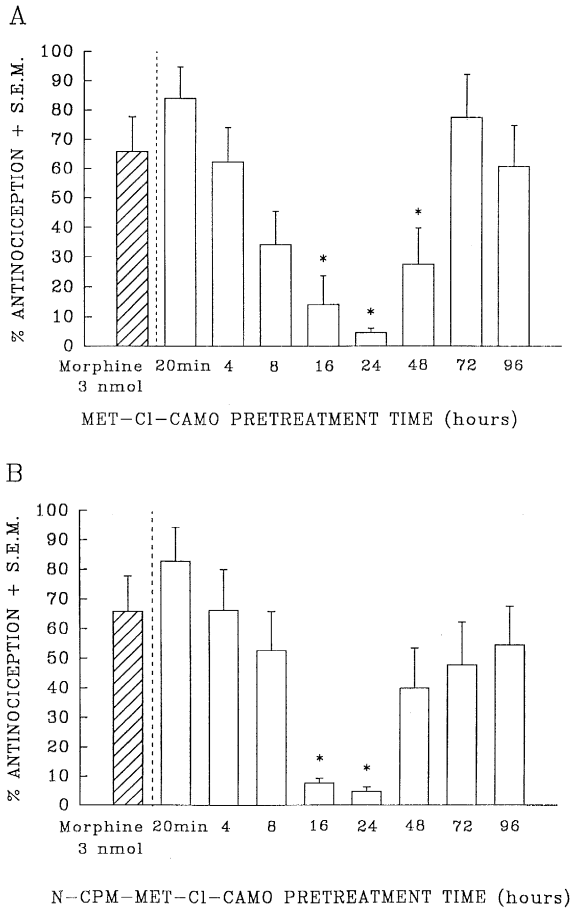

Fig. 4. Antinociceptive effects of i.c.v. morphine (3 nmol, −20 min) in mice pretreated with a single i.c.v. dose of MET-Cl-CAMO (A) or *N*-CPM-MET-Cl-CAMO (B) (1 nmol; 20 min, 4, 8, 16, 24, 72 and 96 h before testing) in the mouse tail-flick assay. * Significant from morphine alone, $P < 0.05$.

N-CPM-MET-Cl-CAMO also displayed high affinity for the δ- and κ-opioid receptors.

Bovine striatal membranes were incubated with varying concentrations of the compounds at 25°C for 15 min, followed by dilution and four centrifugal washes, to detect if MET-Cl-CAMO and *N*-CPM-MET-Cl-CAMO could produce wash-resistant inhibition of opioid binding. Both MET-Cl-CAMO and *N*-CPM-MET-Cl-CAMO produced a concentration-dependent, wash-resistant inhibition of [^{3}H]DAMGO binding (Fig. 2A and B), with a 50% wash-resistant inhibition of the binding of 0.25 nM [^{3}H]DAMGO produced with concentrations of 86 ± 18 nM and 13 ± 3 nM, respectively. Interestingly, a *N*-CPM-MET-Cl-CAMO concentration of 122 ± 43 nM produced a 50% wash-resistant inhibition of the binding of [^{3}H]U69,593, while MET-Cl-CAMO produced no wash-resistant inhibition of κ-opioid binding. Neither compound produced wash-resistant inhibition of δ-opioid receptor binding.

Saturation [^{3}H]DAMGO binding studies demonstrated that membranes pretreated with 100 nM MET-Cl-CAMO had a K_d value for [^{3}H]DAMGO binding of 1.8 ± 0.1

nM, a 3-fold increase over the control K_d value of 0.58 ± 0.05 nM. The B_{max} values for [^{3}H]DAMGO remained unchanged compared to control, 94 ± 9 fmol/mg of protein for control membranes versus 98 ± 11 fmol/mg of protein for membranes pretreated with MET-Cl-CAMO, suggesting that inhibition of binding was due to residual MET-Cl-CAMO.

In contrast, the K_d value for membranes preincubated with 8 nM *N*-CPM-MET-Cl-CAMO remained unchanged, but the B_{max} value for [^{3}H]DAMGO decreased to 41 ± 9 fmol/mg of protein (Fig. 3A). Membranes pretreated with 100 nM *N*-CPM-MET-Cl-CAMO displayed a K_d value 4-fold higher for [^{3}H]U69,593 than the K_d value for untreated control membranes (1.6 ± 0.7 nM versus 0.40 ± 0.01 nM, respectively), while maximal binding of [^{3}H]U69,593 was not significantly changed (16 ± 5.3 fmol/mg of protein for pretreated membranes versus 13 ± 0.5 fmol/mg of protein for control membranes, respectively) (Fig. 3B). The reduction in μ-opioid binding sites without a change in the affinity of the remaining binding sites suggests that *N*-CPM-MET-Cl-CAMO produced wash-resistant inhibition of [^{3}H]DAMGO binding by alkylating the μ-opioid receptor to form a covalent bond. The unchanged maximal binding of [^{3}H]U69,593, accompanied by a 4-fold increase in the K_d value, suggests that when membranes were pretreated with *N*-CPM-MET-Cl-CAMO at a concentration of 100 nM, there was residual drug associated with the membranes, which bound to the κ-opioid receptor in a non-covalent manner.

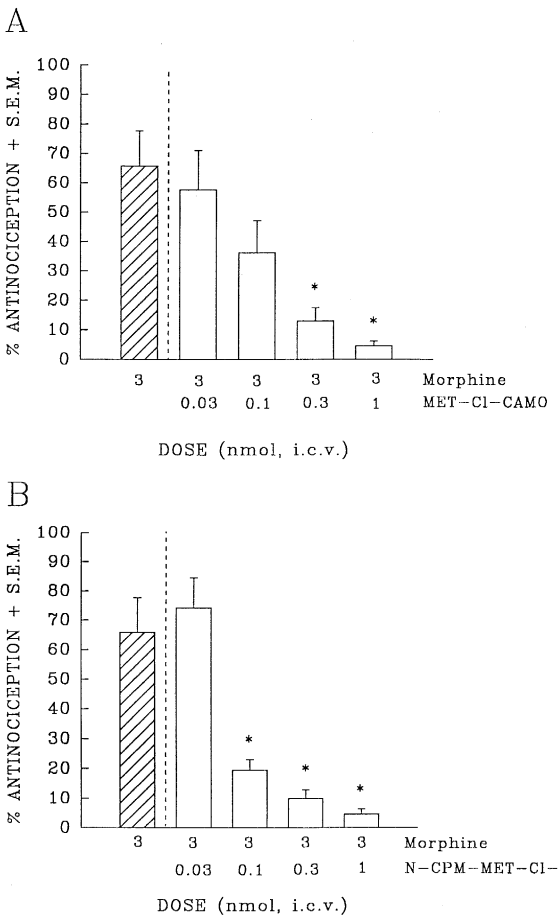

Fig. 5. Antinociceptive effects of i.c.v. morphine sulfate (3 nmol, −20 min) in mice pretreated with a single i.c.v. dose of MET-Cl-CAMO (A) and *N*-CPM-MET-Cl-CAMO (B) (0.03, 0.1, 0.3 or 1 nmol, administered −24 h before testing) in the mouse tail-flick assay. * Significant from morphine alone, $P < 0.01$.

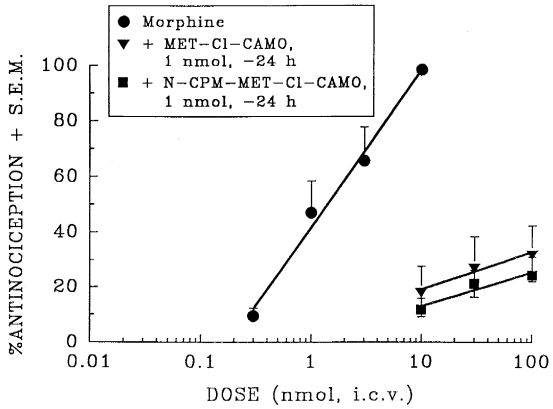

Fig. 6. Dose-response lines for i.c.v. morphine in the absence and presence of i.c.v. MET-Cl-CAMO and *N*-CPM-MET-Cl-CAMO (1 nmol, administered −24 h before testing) in the mouse tail-flick assay.

3.2. Mouse tail-flick assay

No antinociceptive effect was produced by i.c.v. administration of MET-Cl-CAMO or *N*-CPM-MET-Cl-CAMO at doses up to 100 nmol in the mouse 55°C warm-water tail-flick assay. Pretreatment of mice with either MET-Cl-CAMO or *N*-CPM-MET-Cl-CAMO produced a time- and dose-dependent antagonism of morphine-induced antinociception. Significant antagonism produced by 1 nmol of MET-Cl-CAMO appeared 16 h after i.c.v. administration

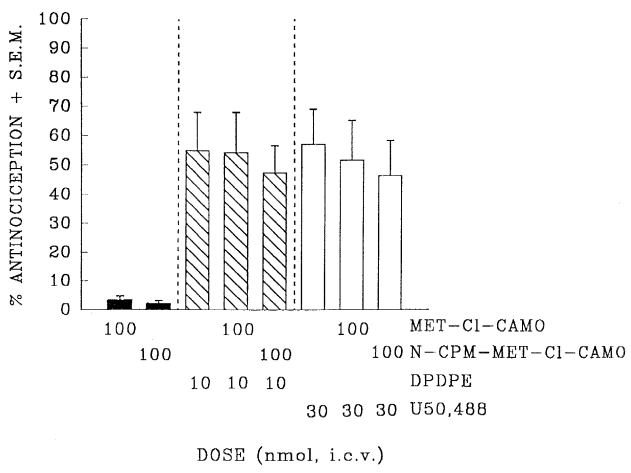

Fig. 7. Antinociceptive effects of i.c.v. DPDPE (10 nmol, −20 min) or U50,488 (30 nmol, −20 min) in mice pretreated without or with a single i.c.v. dose of MET-Cl-CAMO and *N*-CPM-MET-Cl-CAMO (100 nmol, administered −24 h before testing) in the mouse tail-flick assay.

and lasted up to 48 h, while antagonism produced by 1 nmol of *N*-CPM-MET-Cl-CAMO appeared 16 h after i.c.v. administration and lasted up to 24 h (Fig. 4A and B). Maximal antagonism of morphine-induced antinociception by MET-Cl-CAMO and *N*-CPM-MET-Cl-CAMO was observed 24 h after injection. The antagonism of morphine-induced antinociception by MET-Cl-CAMO and *N*-CPM-MET-Cl-CAMO was dose-dependent (Fig. 5A and B). Pretreatment of mice with 1 nmol of either MET-Cl-CAMO or *N*-CPM-MET-Cl-CAMO shifted the dose-response curve of morphine rightward and downward, demonstrating insurmountable antagonism (Fig. 6). Neither MET-Cl-CAMO nor *N*-CPM-MET-Cl-CAMO, administered 24 h before testing in doses up to 100 nmol, antagonized antinociception produced by the δ-opioid receptor agonist DPDPE or the κ-opioid receptor agonist U50,488 (Fig. 7).

4. Discussion

This study investigated the supraspinal opioid-receptor mediated effects of two derivatives of metopon, MET-Cl-CAMO and *N*-CPM-MET-Cl-CAMO, using the mouse warm-water tail-flick assay. Neither compound produced any antinociception in the tail-flick assay, after i.c.v. administration of doses up to 100 nmol. This lack of analgesic activity by a *N*-methyl substituted morphine derivative is consistent with previous findings using the 14β-nitrocinnamoylamino analog MET-CAMO (Jiang et al., 1994), despite the fact that similarly substituted compounds in the morphinan and benzomorphan series are generally agonists (Hellerbach et al., 1966), such as metopon (McLaughlin et al., 1995). Like their nitrocinnamoylamino analogs (Jiang et al., 1994), MET-Cl-CAMO and *N*-CPM-MET-Cl-CAMO produced long-term antagonism of antinociception mediated by the μ-opioid receptor, and shifted the dose-response line for morphine-induced antinociception to the right and downward. This shift in the dose-response line is characteristic of an irreversible opioid receptor antagonist. The delay in the appearance of long-term antagonism of antinociception seen here is consistent with delays reported for most opioid receptor affinity ligands used in vivo. For example, the Michael acceptors β-funaltrexamine and *N*-CPM-MET-CAMO (Jiang et al., 1995) both demonstrated at least 8 h delayed antagonism of morphine analgesia when the lowest dose producing maximal antagonism 24 h after administration was used. This prolonged delay in producing an effect has not been observed when the compounds were incubated with isolated membranes (Jiang et al., 1994). Therefore, the in vivo delay cannot be easily explained chemically. It may be due to the downregulation of the opioid receptor, an event that is known to proceed more slowly than desensitization of the receptor in mediating opioid-induced inhibition of adenylyl cyclase activity (Law et al., 1983).

MET-Cl-CAMO and *N*-CPM-MET-Cl-CAMO were further characterized for opioid receptor affinity with receptor binding assays, and found to bind the μ-opioid receptor with high affinity. Similar to the binding characteristics of the corresponding 14β-*p*-nitrocinnamoylamino compounds (Jiang et al., 1994), the *N*-methyl compound was more selective for the μ-opioid receptor than the *N*-cyclopropylmethyl compound. This finding is consistent with previous studies that have demonstrated higher selectivity for the μ-opioid binding site by morphine derivatives possessing a *N*-methyl constituent versus *N*-cyclopropylmethyl side chains (Pasternak and Hahn, 1980; Jiang et al., 1994). However, *N*-CPM-MET-Cl-CAMO differed from its nitrocinnamoylamino analog, *N*-CPM-MET-CAMO, in proving to be less potent in inhibiting μ-opioid receptor binding in a wash-resistant manner. A 50% wash-resistant inhibition of the binding of 0.25 nM [^{3}H]DAMGO was reported with a *N*-CPM-MET-CAMO concentration of 5.2 ± 0.6 nM (Jiang et al., 1994), a 2.5-fold greater potency in comparison to the chlorocinnamoylamino analog. These differences in wash-resistant inhibition of binding between the chloro- and nitrocinnamoylamino derivatives may be due to the greater electronegativity of the nitro group over that of the chloro group, making it more effective at drawing electrons across the benzene ring of the cinnamoyl constituent. This would leave the carbonyl double bond more susceptible to nucleophilic attack, and the nitrocinnamoylamino compounds, therefore, more reactive in forming covalent bonds. Presumably, this increased reactivity would account for the increased wash-resistant inhibition of [^{3}H]DAMGO binding by the nitrocinnamoylamino derivative in comparison to its chlorocinnamoylamino analog.

It is not clear why the *N*-cyclopropylmethyl compound produced wash-resistant inhibition of [^{3}H]DAMGO binding at much lower concentrations than the *N*-methyl derivative, while both compounds produced similar effects in the mouse warm-water tail flick test. One possible explanation could be differences between the in vivo and in vitro accessibility of the amino acid(s) on the receptor that may form a covalent bond with the compounds. Alternatively, the two compounds may alkylate different amino acids on the μ-opioid receptor. Alkylation of two different amino acids may produce the same long-term inhibition of morphine-induced antinociception. For example, β-funaltrexamine alkylates lysine-233 on the cloned rat μ-opioid receptor (Chen et al., 1996). In contrast, the disulfide-containing affinity ligands 14α,14′β-[dithiobis[(2-oxo-2,1-ethanediyl)imino]]bis(7,8-dihydromorphinone) (TAMO), its *N*-cyclopropylmethyl derivative, *N*-CPM-TAMO, and its *N*-cyclobutylmethyl derivative, *N*-CBM-TAMO, formed a disulfide bond with the receptor as determined by the finding that the disulfide-bond reducing reagent, dithiothreitiol, reversed wash-resistant inhibition of μ-opioid binding produced by these compounds (Jiang et al., 1993b; Archer et al., 1994; Xu et al., in press). β-Funaltrexamine, TAMO, *N*-CPM-TAMO,

and *N*-CBM-TAMO produced long-term antagonism of morphine-induced antinociception (Jiang et al., 1992, 1995; Xu et al., in press). Therefore, despite the fact that β-funaltrexamine binds to a lysine residue and the disulfide-containing TAMO derivatives bind to a thiol group, both types of compounds produced the same long-term antagonism of morphine-induced antinociception. With the cinnamoylamino derivatives, the *N*-methyl derivative, MET-Cl-CAMO, and the *N*-cyclopropylmethyl derivative, *N*-CPM-MET-Cl-CAMO, may bind to different amino acids on the receptor. Alkylation of the receptor produces long-term antagonism of antinociception mediated by μ-opioid receptors. However, unless the alkylation of the receptor alters the [^{3}H]DAMGO binding site, changes in radioligand binding would not be observed.

Both the binding and the analgesic studies indicate that *N*-CPM-MET-Cl-CAMO forms a covalent bond with the μ-opioid receptor. The suggested covalent bond formed between *N*-CPM-MET-Cl-CAMO and the μ-opioid receptor may be produced by the chlorocinnamoylamino group acting as a Michael acceptor, undergoing nucleophilic addition to eligible substrates in the receptor, such as thiol or primary amino groups found in cysteine or lysine amino acids, respectively. The extracellular amino-terminal tail of the rat μ-opioid receptor is reported to have one lysine and five cysteine amino acids, providing six potential sites for the formation of a covalent bond with a Michael acceptor (Thompson et al., 1993). Previous studies have suggested that the extracellular amino-terminal tail may play a role in antagonist binding and opioid receptor selectivity, supporting the idea that the antagonist *N*-CPM-MET-Cl-CAMO might interact with the amino-terminal tail of the μ-opioid receptor (Kong et al., 1994; Xue et al., 1995). Alternatively, *N*-CPM-MET-Cl-CAMO may bind irreversibly to the third extracellular loop of the μ-opioid receptor. The third extracellular loop between transmembrane regions 6 and 7 of the rat μ-opioid receptor possesses a lysine residue whereas the δ- and κ-opioid receptors lack this residue (Thompson et al., 1993). This difference between receptors could account for the irreversible binding of *N*-CPM-MET-Cl-CAMO to the μ-opioid receptor, measured here as a reduction in the maximal binding of [^{3}H]DAMGO to neuronal membranes preincubated with *N*-CPM-MET-Cl-CAMO, but not the κ-opioid receptor, as maximal [^{3}H]U69,593 binding remained unchanged. Support for this mechanism comes from work with [^{3}H]β-funaltrexamine, which required the region from the middle of the third intracellular loop to the C-terminus of the μ-opioid receptor to bind irreversibly (Chen et al., 1995). Moreover, the ability of lysine to provide a substrate for a Michael addition reaction was shown by subsequent work where [^{3}H]β-funaltrexamine formed a covalent bond with lysine-233 in the second extracellular loop of the cloned rat μ-opioid receptor (Chen et al., 1996). Studies have further demonstrated that the nitrocinnamoylamino derivative, [^{3}H]*N*-CPM-CACO, bound covalently to the μ-opioid receptor in bovine striatal membranes (McLaughlin et al., in press).

In conclusion, these results suggest that MET-Cl-CAMO and *N*-CPM-MET-Cl-CAMO act like their nitrocinnamoylamino analogs in being devoid of agonist activity but producing μ-opioid receptor selective, long-term antagonism of antinociception in the mouse warm-water tail-flick test. However, variation in potency and κ-opioid receptor binding suggest that there are important differences between the chlorocinnamoylamino and nitrocinnamoylamino compounds.

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