Nitrocinnamoyl and Chlorocinnamoyl Derivatives of Dihydrocodeinone: In Vivo and In Vitro Characterization of μ-Selective Agonist and Antagonist Activity

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ABSTRACT

Two 14β-p-nitrocinnamoyl derivatives of dihydrocodeinone, 14β-(p-nitrocinnamoylamino)-7,8-dihydrocodeinone (CACO) and N-cyclopropylmethyl-14β-(p-nitrocinnamoylamino)-7,8-dihydrocodeinone (N-CPM-CACO), and the corresponding chlorocinnamoylamino analogs, 14β-(p-chlorocinnamoylamino)-7,8-dihydrocodeinone (CAM) and N-cyclopropylmethyl-14β-(p-chlorocinnamoylamino)-7,8-dihydrocodeinone (MC-CAM), were tested in opioid receptor binding assays and the mouse tail-fllick test to characterize the opioid affinity, selectivity, and antinociceptive properties of these compounds. In competition binding assays, all four compounds bound to the μ opioid receptor with high affinity. When bovine striatal membranes were incubated with any of the four dihydrocodeinones, binding to the μ receptor was inhibited in a concentration-dependent, wash-resistant manner. Saturation binding experiments demonstrated that the wash-resistant inhibition of μ binding was due to a decrease in the Bmax value for the binding of the μ-selective peptide [3H][D-Ala2,MePhe4,Gly(ol)5]enkephalin and not a change in the Kd value, suggesting an irreversible interaction of the compounds with the μ receptor. In the mouse 55°C warm water tail-flick test, both CACO and N-CPM-CACO acted as short-term μ-selective agonists when administered by i.c.v. injection, whereas CAM and MC-CAM produced no measurable antinociception at doses up to 30 nmol. Pretreatment of mice for 24 h with any of the four dihydrocodeinone derivatives produced a dose-dependent antagonism of antinociception mediated by the μ but not the δ or κ receptors. Long-term antagonism of morphine-induced antinociception lasted for at least 48 h after i.c.v. administration. Finally, shifts in the morphine dose-response lines after 24-h pretreatment with the four dihydrocodeinone compounds suggest that the nitrocinnamoylamino derivatives may produce a greater magnitude long-term antagonism of morphine-induced antinociception than the chlorocinnamoylamino analogs.

The synthesis of ligands acting as long-term opioid antagonists has led to advances in opioid receptor pharmacology (Portoghese et al., 1980; Rice et al., 1983; Bidlack et al., 1993; Archer et al., 1994). The capability of a ligand to act as an irreversible opioid antagonist is essentially based on two features: 1) the affinity of the ligand for the binding site of the opioid receptor; and 2) the ability of the ligand to form a permanent, covalent bond with the opioid receptor. Explained kinetically, the first factor influences the formation of the receptor-ligand complex, measured as rate k1, and the second the rate at which the ligand then binds covalently to the receptor, rate k2 (Liu-Chen et al., 1990). Presumably, this covalent bond produces irreversible antagonism of opioid-induced antinociception, because the ligand permanently blocks the opioid receptor binding site from other opioids.

One successful strategy in the synthesis of irreversible opioid antagonists involves using opioids with progressively higher affinity and selectivity for the opioid receptor, mated to α,β-
unsaturated carbonyl groups with better electron-withdrawing groups on the β-carbon. This carbonyl forms a covalent bond through a Michael addition reaction with eligible nucleophiles in the opioid receptor, such as the thiols found in cysteine or the primary amino group found in lysine. An early attempt added the α, β-unsaturated carbonyl fumaramate to the opioid antagonist naltrexone in the C-6 position to yield β-fumaltrexamine (β-FNA) (Portoghese et al., 1980). As expected, β-FNA produced long-term antagonism of morphine-induced antinociception (Ward et al., 1982; Liu-Chen and Phillips, 1987) and was shown to form an irreversible, covalent bond to the amino acid lysine-233 of the rat μ opioid receptor (Chen et al., 1996). However, use of the antagonistic properties of β-FNA is complicated by short-term κ agonist effects, the need for high doses to produce long-term antagonism, and delays in the onset of antagonism to μ receptor-mediated antinociception, prompting a search for compounds that act more selectively as irreversible μ opioid antagonists with greater ease of use (Jiang et al., 1995). Nitrocinnamoylamino groups were added to the 14-β position of metopon derivatives to yield 5β-methyl-14β-(p-nitrocinnamoylamino)-7,8-dihydromorphinone (MET-CAMO) and N-cyclopropylmethyl-5β-methyl-14β-(p-nitrocinnamoylamino)-7,8-dihydromorphinone (N-CPM-MET-CAMO; Sebastian et al., 1993). Subsequent work ascribed the observed μ-selective irreversible antagonism of morphine-induced antinociception to the α,β-unsaturated carbonyl constituents acting as Michael acceptors to bind covalently to the μ receptor (Jiang et al., 1994). Similar results were seen in a separate study with a chlorocinnamoylamino analog, N-CPM-MET-Cl-CAMO (McLaughlin et al., 1997b). However, the cinnamoylamino group has not been directly observed to react with thiol groups as expected of an α,β-unsaturated carbonyl; when incubated with N-acetylcysteine, N-CPM-MET-CAMO was recovered unchanged (Sebastian et al., 1993). Moreover, work performed with tritiated C,C-CAM, a normorphinone derivative with the same chlorocinnamoylamino side chain as N-CPM-MET-Cl-CAMO, demonstrated long-term antagonism of morphine-induced antinociception without evidence of covalent labeling of the μ opioid receptor in subsequent protein isolation experiments (Zernig et al., 1995, 1996). Given these conflicting data, it remains unclear whether 14β-cinnamoylamino derivatives of dihydromorphinone produce long-term antagonism through an irreversible covalent bond in vivo. Furthermore, it remains unclear whether the nitro- or the chloro-cinnamoylamino group is more effective in producing long-term opioid receptor antagonism, because no direct comparison in a single study has been made to date. Finally, new irreversible opioid receptor antagonists may prove useful in research if they possess greater selectivity for the opioid receptor and prove clinically useful as well, because the irreversible μ receptor antagonists reduce self-administration of addictive drugs (Woods et al., 1995; Martin et al., 1995; Archer et al., 1996; Krishnan-Sarin et al., 1998).

The present work studied four derivatives of dihydrocodeinone containing the nitrocinnamoylamino or chlorocinnamoylamino α,β-unsaturated carbonyl constituents in the 14β position (Fig. 1). The study characterized the opioid affinity, selectivity, and efficacy of 14β-(p-nitrocinnamoylamino)-7,8-dihydrocodeinone (CACO), N-cyclopropylmethylnor-14β-(p-nitrocinnamoylamino)-7,8-dihydrocodeinone (N-CPM-CACO), 14β-(p-chlorocinnamoylamino)-7,8-dihydrocodeinone (CAM), and N-cyclopropylmethylnor-14β-(p-chlorocinnamoylamino)-7,8-dihydrocodeinone (MC-CAM) in competition binding assays and analgesic assays to ascertain whether the dihydrocodeinones containing α,β-unsaturated carbonyl groups could produce μ-selective irreversible opioid antagonism. It is important to note MC-CAM has been reported previously to be a partial μ agonist and irreversible antagonist of morphine-induced antinociception in the mouse vas deferens preparation and in rodent and monkey analgesic assays (Aceto et al., 1989; Woods et al., 1995). However, the compounds CAM and MC-CAM are structurally similar to CACO and N-CPM-CACO, differing only in containing a 14β-p-chlorocinnamoylamino rather than a 14β-p-nitrocinnamoylamino group, respectively, allowing a direct comparison between cinnamoylamino groups to determine which one has the greatest potency in producing irreversible antagonism of morphine-induced antinociception.

Materials and Methods

Synthesis of CAM, MC-CAM, CACO, and N-CPM-CACO

The nitrocinnamoylamino dihydrocodeinones, CACO and N-CPM-CACO, were synthesized as described previously (Sebastian et al., 1993). The synthesis of the chlorocinnamoylamino dihydrocodeinone analogs, CAM and MC-CAM, was carried out in an identical manner to the procedure described in Sebastian et al. (1993), except that p-chlorocinnamoyl chloride was substituted for the p-nitrocinnamoyl chloride in the reaction.

In Vitro Studies

Opioid Binding to Bovine Striatal Membranes. Bovine striatal membranes were prepared as described previously (Jiang et al., 1994). The affinity and selectivity of the compounds CACO, N-CPM-CACO, CAM, and MC-CAM for the multiple opioid receptors was determined by incubating the membranes with radiolabeled ligands and 12 different concentrations of the compounds 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-selective peptide [H]D-Ala2,D-Pen5 enkephalin ([D-Ala2,(Me)Phe4,Gly-ol]5 enkephalin (DAMGO) and the κ-selective ligand [H]5a,7a,8β-(→)-N-methyl-N-(1-pyrrolidinyl)-1-oxaspiro(4,5)dec-8-yl benzeneacetamide (U69,593). A 4-h incubation was used with the δ-selective peptide [H]D-Pen2, p-Cl-phenylalanine1, D-Pen5 enkephalin (pCIP-DPDE). To determine the IC50 values for the inhibition of binding by the compounds, the final concentrations of [H]DAMGO, [H]Cl-DPDE, and [H]U69,593 were 0.25, 0.2, and 1 nM, respectively. Nonspecific binding was measured by inclusion of 10 μM naloxone. Binding was terminated by filtering the samples through Schleicher & Schuell No. 32 glass fiber filters (Keene, NH) using a Brandel 48-well cell harvester. Filters were soaked for at least 60 min in 0.25% polyethyleneimine for [H]Cl-DPDE and [H]U69,593 binding experiments. After filtration, filters were washed three times. 

![Fig. 1. Structures of CACO, N-CPM-CACO, CAM, and MC-CAM.](image-url)
with 3 ml of cold 50 mM Tris-HCl, pH 7.5, and were counted in 2 ml of Ecoscint A scintillation fluid.

**Wash-Resistant Inhibition of Opioid Binding by Affinity Ligands.** The 14β-p-cinnamoymalino side chain of the compounds may bind covalently to the opioid receptor. Experiments measuring wash-resistant inhibition of opioid binding were performed to detect potential covalent binding. To determine the concentration of the compounds CAM, MC-CAM, CACO, and N-CPM-CACO needed to obtain wash-resistant inhibition of opioid binding, bovine striatal membranes, 10 mg of protein, were incubated with concentrations of the compounds CAM, ranging from 0.25 to 8 nM; N-CPM-CAM, ranging from 2 to 75 nM; and CACO and N-CPM-CACO, ranging from 3 to 200 nM at 25°C for 15 min, in a final volume of 2 ml. The contents of the tubes were then diluted to 40 ml with cold 50 mM Tris-HCl, pH 7.5, and centrifuged at 39,000 rpm for 20 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 receptor affinity or reduced the number of the opioid binding sites, 10 mg of membrane protein were incubated with either 50 nM CACO, 50 nM N-CPM-CACO, 20 nM CAM, or 64 nM MC-CAM for 15 min in a final volume of 2 ml 50 mM Tris-HCl, pH 7.5, followed by four centrifugal washes. 

**In Vivo Studies.** Animals. All antinociceptive experiments used male, ICR mice (20–24 g; Harlan Sprague-Dawley, Indianapolis, IN). 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.

**Injection Techniques.** i.c.v. 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.

**Tail-Flick Assay.** The thermal nociceptive stimulus was 55°C water, with the latency to tail-flick or withdrawal taken as the endpoint (Vaugt and Takemori, 1979). After determining control latencies, the mice received graded i.c.v. doses of opioid agonists or antagonists at various times. Morphine sulfate, [D-Pen2, D-Pen5]-enkephalin (DPDPE), (trans)-3,4-dichloro-N-methyl-N-[2-(1-pyryrolidinyl)cyclohexyl]benzeneacetamide methane-sulfonate hydrate (U50,488), and the compounds CACO, N-CPM-CACO, CAM, and MC-CAM were given as single i.c.v. injections with antinociceptive effect measured 20 min after injection unless otherwise stated. In the antagonist study, various doses of the compounds CACO, N-CPM-CACO, CAM, and MC-CAM were given as a single pretreatment at 0, 4, 8, 16, 24, 48, and 72 h before testing. In the receptor selectivity studies, either the κ-selective antagonist, nor-binaltorphimine (nor-BNI), or the δ-selective antagonist, N,N-dialyl-Tyr-Alb-Aib-Phe-Leu-ΟΗ (where Aib is α-aminoisobutyric acid) (ICI 174,864), were each given with the agonist in the same injection. β-FNA, the μ-selective antagonist, was injected 24 h before agonist injection. A cut-off time of 15s 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 that 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.

**Chemicals.** [3H]DAMGO (60 Ci/mmol) and [3H]U69,593 (64 Ci/mmol) were purchased from Amersham (Arlington Heights, IL). [3H]pCl-DPDPE (48.6 Ci/mmol) was obtained from New England Nuclear (Boston, MA). Morphine sulfate was purchased from Mallinkrodt Chemical Company (St. Louis, MO). DPDPE, U50,488H, nor-BNI, ICI 174,864, and β-FNA were purchased from Research Biochemicals International (Natick, MA).

In the mouse tail-flick assay, CACO, N-CPM-CACO, nor-BNI, and ICI 174,864 were dissolved in 20% dimethyl sulfoxide, a concentration that did not produce any detectable effect. CAM, MC-CAM, β-FNA, DPDPE, and U50,488 and morphine sulfate were dissolved in distilled water.

**Statistics.** IC50 values were calculated by least-squares fit to a log-logistic-probit analysis. Saturation [3H]DAMGO binding data were analyzed by nonlinear regression analysis using the LIGAND program (Muson and Rodbard, 1980). All dose-response lines were analyzed, using the regression methods described by Tallarida and Murray (1986). Regression lines, D50 (dose producing 50% antinociception) values and 95% confidence limits were determined with each individual data point (Tallarida and Murray, 1986). All data points shown are the mean of 7–10 mice, with S.E.M. represented by error bars.

**Results.**

**In Vitro Studies.**

**Competitive and Wash-Resistant Inhibition of Opioid Binding.** The 14β-p-cinnamoymalino-containing compounds, CACO, N-CPM-CACO, CAM, and MC-CAM (Fig. 1), all demonstrated higher affinity for the μ-, rather than δ- and κ-opioid binding sites, as determined by comparison of their IC50 values for the inhibition of μ-, δ-, and κ-opioid binding to bovine striatal membranes (Table 1). The N-methyl compound displayed higher affinity for the μ-opioid receptor than their N-cyclopropylmethyl analogs in both cases. Because the 14β-cinnamoymalino side chain has the potential to bind covalently to the receptor, it cannot be assumed to bind at equilibrium, and therefore only IC50 values are reported. To determine whether the 14β-cinnamoymalino-containing compounds bind covalently to the opioid receptor, bovine striatal membranes were pretreated with the four compounds, and wash-resistant inhibition of opioid binding, indicative of a covalent bond, was measured. Bovine striatal membranes were incubated with varying concentrations of the affinity ligands at 25°C for 15 min, followed by dilution and four centrifugal washes, to detect wash-resistant inhibition of the binding of 0.25 nM [3H]DAMGO produced by CACO, N-CPM-CACO, CAM, or MC-CAM. All four compounds, CACO, N-CPM-CACO, CAM, and MC-CAM, 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).

**TABLE 1**

<table>
<thead>
<tr>
<th>Ligands</th>
<th>IC50 (nM ± S.E.M.)</th>
<th>μ</th>
<th>δ</th>
<th>κ</th>
</tr>
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<tbody>
<tr>
<td>CACO</td>
<td>0.46 ± 0.003</td>
<td>4.2 ± 1.3</td>
<td>19 ± 2.8</td>
<td></td>
</tr>
<tr>
<td>N-CPM-CACO</td>
<td>1.1 ± 0.007</td>
<td>18 ± 4.4</td>
<td>9.8 ± 0.61</td>
<td></td>
</tr>
<tr>
<td>CAM</td>
<td>0.12 ± 0.01</td>
<td>0.75 ± 0.15</td>
<td>1.1 ± 0.20</td>
<td></td>
</tr>
<tr>
<td>MC-CAM</td>
<td>1.4 ± 0.12</td>
<td>12 ± 1.5</td>
<td>14 ± 3.5</td>
<td></td>
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</table>

| Membranes were incubated with varying concentrations of compounds CACO, N-CPM-CACO, CAM, and MC-CAM in presence of either 0.25 nM [3H]DAMGO, 0.2 nM [3H]pCl-DPDPE, or 1 nM [3H]U69,593 to measure binding to μ-, δ-, and κ-sites, respectively. After equilibrium binding was reached, membranes were filtered onto glass-fiber filters as described in Materials and Methods. Data are expressed as mean IC50 value ± S.E.M. for three determinations performed in triplicate.
pounds produced a concentration-dependent, wash-resistant inhibition of [3H]DAMGO binding (Fig. 2). The concentrations that produced a 50% inhibition of 0.25 nM [3H]DAMGO binding were 11 ± 1 nM CACO, 22 ± 0.7 nM N-CPM-CACO, 2.1 ± 0.2 nM CAM, and 19 ± 2.1 nM MC-CAM. Neither CACO, N-CPM-CACO, nor CAM produced wash-resistant inhibition of [3H]pCl-DPDPE and [3H]U69,593 binding in the concentration range which produced ~80% inhibition of µ binding for each affinity ligand (Fig. 2, A–C). However, high pretreatment concentrations of MC-CAM produced modest inhibition of [3H]pCl-DPDPE and [3H]U69,593 binding in a wash-resistant manner (Fig. 2D).

Saturation Binding of [3H]DAMGO to Bovine Striatal Membranes Pretreated with Cinnamoylamino-Containing Compounds. To determine whether the four cinnamoyl-containing compounds produced the wash-resistant inhibition of [3H]DAMGO binding (Fig. 2). The concentrations that produced a 50% inhibition of 0.25 nM [3H]DAMGO binding were 11 ± 1 nM CACO, 22 ± 0.7 nM N-CPM-CACO, 2.1 ± 0.2 nM CAM, and 19 ± 2.1 nM MC-CAM. Neither CACO, N-CPM-CACO, nor CAM produced wash-resistant inhibition of [3H]pCl-DPDPE and [3H]U69,593 binding in the concentration range which produced ~80% inhibition of µ binding for each affinity ligand (Fig. 2, A–C). However, high pretreatment concentrations of MC-CAM produced modest inhibition of [3H]pCl-DPDPE and [3H]U69,593 binding in a wash-resistant manner (Fig. 2D).

Table 2 summarizes the saturation binding results to untreated, control bovine striatal membranes or pretreated with CACO, N-CPM-CACO, CAM, or MC-CAM. Membranes were incubated with 50 nM CACO, 50 nM N-CPM-CACO, 8 nM CAM, or 64 nM MC-CAM for 15 min at 25°C. Control samples lacked compound in preincubation. Membranes were then diluted with 50 mM Tris-HCl, pH 7.5, followed by four centrifugal washes. Membranes were finally resuspended in 50 mM Tris-HCl, pH 7.5, and [3H]DAMGO binding at concentrations ranging from 0.025 to 3.2 nM was measured as described in Materials and Methods. Data are expressed as mean value ± S.E.M. for three determinations performed in triplicate.

<table>
<thead>
<tr>
<th>Ligands</th>
<th>$K_d$ (nM)</th>
<th>$B_{max}$ (fmol/mg of protein)</th>
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<tbody>
<tr>
<td>Control</td>
<td>0.63 ± 0.05</td>
<td>130 ± 4</td>
</tr>
<tr>
<td>50 nM CACO</td>
<td>0.52 ± 0.14</td>
<td>29 ± 9</td>
</tr>
<tr>
<td>50 nM N-CPM-CACO</td>
<td>0.46 ± 0.06</td>
<td>25 ± 1</td>
</tr>
<tr>
<td>8 nM CAM</td>
<td>1.1 ± 0.11</td>
<td>48 ± 3</td>
</tr>
<tr>
<td>64 nM MC-CAM</td>
<td>2.4 ± 0.43</td>
<td>73 ± 7</td>
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Fig. 2. Wash-resistant inhibition of opioid binding produced by preincubation of bovine striatal membranes with CACO, N-CPM-CACO, CAM, or MC-CAM. Membrane protein at a concentration of 10 mg was incubated with varying concentrations of CACO (A), N-CPM-CACO (B), CAM (C), or MC-CAM (D) at 25°C for 15 min, followed by four centrifugal washes. Binding of 0.25 nM [3H]DAMGO, 0.2 nM [3H]pCl-DPDPE, and 1 nM [3H]U69,593 to 0.2 ml of membranes was measured as described in Materials and Methods. Data are presented as the mean percentage of control binding ± S.E.M. from three or more experiments performed in triplicate.
Antinociceptive Effects of Affinity Ligands in Mouse 55°C Warm Water Tail-Flick Assay. No antinociceptive effect was produced by i.c.v. administration of CAM or MC-CAM at doses up to 30 nmol in the mouse tail-flick assay (data not shown). However, antinociception was produced in a dose-dependent manner by i.c.v. administration of CACO and N-CPM-CACO (Fig. 3A and B, respectively). The antinociceptive D_{50} values of CACO and N-CPM-CACO (and 95% confidence limits) were 1.8 (1.1–3.1) nmol and 0.6 (0.4–1.0) nmol, respectively (Fig. 3). This antinociception was maximal after 20 min and lasted up to 2 h (data not shown). Moreover, the antinociception induced by either nitrocinnamoylamino compound was inhibited by 24-h pretreatment with the \( \mu \)-selective antagonist \( \beta \)-FNA but not the \( \delta \)-selective antagonist ICI 174,864 or the \( \kappa \)-selective antagonist nor-BNI (Fig. 3). Together, these data demonstrate that the chlorocinnamoyl compounds CAM and MC-CAM lack agonist activity in the 55°C warm-water tail-flick test, whereas the corresponding nitrocinnamoyl analogs, CACO and N-CPM-CACO, produced antinociception by acting as short-term \( \mu \) agonists.

Antagonist Effects Produced by Affinity Ligands Against Opioid-Induced Antinociception in Mouse 55°C Warm Water Tail-Flick Assay. Pretreatment of mice with either CACO, N-CPM-CACO, CAM, or MC-CAM produced a time- and dose-dependent antagonism of morphine-induced antinociception. Significant antagonism produced by 1 nM CACO, N-CPM-CACO, or CAM appeared 16 h after i.c.v. administration and lasted up to 48 h (Fig. 4, A–C). Similar results were obtained with MC-CAM, although at 10-fold higher doses of pretreatment (Fig. 4D). Maximal antagonism of morphine-induced antinociception by the affinity ligands was dose dependent (Fig. 5A and B). Pretreatment of mice for 24 h with 1-nmol CACO or N-CPM-CACO (Fig. 6A) or 1-nmol CAM or 10-nmol MC-CAM (Fig. 6B) shifted the dose-response curve of morphine rightward, demonstrating antagonism. Additionally, CACO and N-CPM-CACO shifted the dose-response curve of morphine downward as well as rightward (Fig. 6A), suggesting that the antagonism produced by the nitrocinnamoylamino compounds was insurmountable. In contrast, none of the four affinity ligands antagonized the antinociception produced by the \( \delta \)-selective agonist DPDPE or the \( \kappa \)-selective agonist U50,488 at doses up to 100 nmol, either when coadministered with the agonists or in mice after 24-h pretreatment (data not shown.)

Discussion

This study used the mouse 55°C warm-water tail-flick assay to investigate the supraspinal, opioid-mediated effects of four derivatives of dihydrocodeinone, CACO, N-CPM-CACO, CAM, and MC-CAM. Although the nitrocinnamoylamino analogs displayed a dose-dependent, short-term antinociception, neither chlorocinnamoylamino derivative produced antinociception in the tail-flick assay after i.c.v. administration of doses up to 30 nmol. This lack of analgesic activity is consistent with previous investigations of morphinone derivatives containing 14\( \beta \)-p-chlorocinnamoylamino constituents (McLaughlin et al., 1997b), but work done by others has suggested that MC-CAM acts as a partial \( \mu \) agonist of long duration, using the phenylquinone-induced writhing assay in mice (Aceto et al., 1989) and the 50°C tail-withdrawal assay with rhesus monkeys (Woods et al., 1995; Butelman et al., 1996). However, these previous characterizations of MC-CAM were based on peripheral administration rather than the i.c.v. route used here, which could conceivably account for the differences in agonist activity. Moreover, the difference in tail-flick assay temperature used here to detect agonist effects, 55°C versus 50°C in the study by Butelman et al. (1996), may explain the differences in MC-CAM results. A 5-degree increase in the tail-flick assay temperature, from 50°C to 55°C, was shown previously to produce a loss of MC-CAM analgesic activity in rhesus monkeys (Butelman et al., 1996).

Both nitrocinnamoyl compounds tested produced long-term antagonism of antinociception mediated by the \( \mu \) receptor and shifted the dose-response line for morphine-induced antinociception to the right and downward. This shift in the morphine antinociceptive response is characteristic of an irreversible opioid receptor antagonist (Woods et al., 1985) and is consistent with previous work done with the cinnamoyl compounds MET-CAMO, N-CPM-MET-CAMO, N-CPM-MET-C1-CAMO, and C-CAM (Comer et al., 1992; Jiang et al., 1994; McLaughlin et al., 1997b). Likewise, the 16-h delay in

Fig. 3. Dose-response lines for i.c.v. CACO (A) and N-CPM-CACO (B) in the presence or absence of either i.c.v. ICI174,864 (4 nmol), nor-BNI (1 nmol), or \( \beta \)-FNA (20 nmol, –24 h) in the mouse tail-flick test. Testing occurred 20 min after the injection of the affinity ligands and reversible antagonists.
the appearance of long-term antagonism of morphine-induced antinociception demonstrated here is consistent with delays reported with other irreversible opioid antagonists used (Jiang et al., 1995). The reason for this delay in the onset of opioid antagonism is unclear but is dose dependent. It has been suggested that MC-CAM undergoes metabolism to become a long-term opioid antagonist (Woods et al., 1995; Husband et al., 1998). This explanation seems unlikely because the same delays in the onset of opioid antagonism are seen here with central administration of the cinnamoyl compounds tested. The delay in the onset of antagonism of morphine-induced antinociception is dependent on the dose of the irreversible compound. This phenomenon was observed with β-FNA and N-CPM-TAMO, in addition to the cinnamoylamino compounds (Jiang et al., 1995). Moreover, in vitro studies with [3H]β-FNA and [3H]N-CPM-CACO suggest that they bind covalently to the μ-opioid receptor within minutes, discounting a chemical explanation of the in vivo delay (Liu-Chen et al., 1990; McLaughlin et al., 1997a). A more feasible explanation may lie in the presence of a sizable opioid receptor reserve and the turnover of the opioid receptor from the cell membrane, an event known to proceed over a time course of several hours (Fantozzi et al., 1981; Law et al., 1983; Arden et al., 1995). The putative irreversible opioid antagonists might exert their effects only after binding to a significant fraction of the receptor reserve, limited in receptors available to bind as dictated by the rate of receptor turnover, possibly producing the delay.

Because all four compounds demonstrated good affinity for the μ receptor in competition binding assays, it is interesting that after a 24-h pretreatment, a 1-nmol dose of CACO, N-CPM-CACO, or CAM all produced long-term antagonism of opioid-induced antinociception in the tail-flick assay, whereas MC-CAM required a 10-fold greater dose to produce the same effect. Moreover, in contrast to previous findings, the chlorocinnamoylamino dihydrocodeine, CAM, was more effective at producing wash-resistant inhibition of [3H]DAMGO binding to bovine striatal membranes than the other three cinnamoylamino compounds tested, producing a 50% wash-resistant inhibition of the binding of 0.25 nM [3H]DAMGO at a 10-fold lower concentration. This finding is inconsistent with the only other comparison of chloro- and nitrocinnamoylamino groups available, between MET-Cl-
CAMO and N-CPM-MET-CI-CAMO (McLaughlin et al., 1997b) and MET-CAMO and N-CPM-MET-CAMO (Jiang et al., 1994), respectively. Comparison of these metopon derivatives across the two studies suggested that the nitrocinnamoylamino side chain produced a more potent irreversible antagonism of morphine-induced antinociception than the chlorocinnamoylamino group, and that the \(N\)-methyl derivatives were much less effective than the \(N\)-cyclopropylmethyl derivative in producing irreversible opioid antagonism. The discrepancy in potency found in the present study might be due to the difference between dihydromorphinone and dihydrocodeinone binding to the opioid receptor. Otherwise, however, the findings suggest little difference between a chloro- or nitro-withdrawing group on the cinnamoylamino unsaturated carbonyl constituent for the production of irreversible opioid antagonism. These findings further emphasize the previous conclusion that the placement of the electron-withdrawing groups in the \textit{para} position of the cinnamoylamino substituent increases the potency of irreversible opioid antagonist activity, whereas comparable substituents in the \textit{ortho} and \textit{meta} positions produce less potent, or ineffective, irreversible opioid antagonists (Lewis et al., 1988; Nieland et al., 1995).

Reversible opioid partial agonists such as cyclazocine, nalbuphine, and buprenorphine have been suggested for therapeutic use in the treatment of heroin abuse, because they produce a more mild series of withdrawal symptoms and reduce drug craving in comparison to untreated human subjects (Martin et al., 1965; Fink et al., 1972). Similarly, MC-CAM has been shown to moderate the severity of opioid withdrawal symptoms in monkeys, suggesting that irreversible opioid antagonists might also have therapeutic value in the treatment of drug abuse (Woods et al., 1995). This idea is supported by animal studies showing a decrease in opioid self-administration rates after pretreatment with the irreversible opioid antagonists \(\beta\)-FNA, N-CPM-TAMO, or C-CAM (Martin et al., 1995; Archer et al., 1996; Zernig et al., 1997). Additionally, 14\(\beta\)-(thioglycolamido)-7,8-dihydro-N(4-cyclobutylmethyl)-morphinone suppressed cocaine self-administration rates in rats as well as heroin (Archer et al., 1996), and \(\beta\)-FNA was found to suppress alcohol intake in rats (Krishnan-Sarin et al., 1998), suggesting that opioid short-
term agonists/long-term antagonists may offer a generic therapeutic value in treating many types of drug abuse, possibly through interaction with the dopamine reward pathway (Wise and Bozarth, 1987; Spanagel et al., 1992; Negus et al., 1993; Zernig and Fibiger, 1998). As CACO and N-CPM-CACO were demonstrated to be short-term μ agonists and long-term irreversible antagonists of μ-opioid-mediated antinociception, these two compounds might serve as effective therapeutics in treating drug abuse, with the μ agonist effects encouraging greater treatment compliance.

In conclusion, derivatives of dihydrocodeine containing nitrocinna-myolaminio (CACO and N-CPM-CACO) or chlorocinnamonolynmo constituents (CAM and MC-CAM) produced approximately equivalent long-term antagonism of morphine-induced antinociception. However, CACO and N-CPM-CACO also acted as short-term μ agonists, suggesting that these nitrocinna-myolaminio dihydrocodeine compounds may have therapeutic value in the treatment of drug abuse.

References


