ELSEVIER

European Journal of Pharmacology 337 (1997) 27–34

ejp

Clocinnamox dose-dependently antagonizes morphine-analgesia and [^{3}H]DAMGO binding in rats

Carol A. Paronis [a,*], James H. Woods [a,b]

[a] *Department of Pharmacology, University of Michigan, Ann Arbor, MI, USA*
[b] *Department of Psychology, University of Michigan, Ann Arbor, MI, USA*

Received 4 August 1997; accepted 22 August 1997

Abstract

Clocinnamox is a long-lasting, nonequilibrium, μ-opioid receptor antagonist in mice and monkeys. The present studies examined the in vivo and ex vivo effects of clocinnamox in rats. Under control conditions, morphine dose-dependently increased tail-withdrawal latencies from 50°C water, with a mean ED_{50} of 7.3 ± 1.1 mg/kg. Clocinnamox antagonized the antinociceptive effects of morphine. 1.0 mg/kg clocinnamox displaced the morphine dose–response curve 4-fold to the right of the control curve and 10 mg/kg clocinnamox eliminated morphine's antinociceptive effects at doses up to 1000 mg/kg for at least seven days. There was a gradual recovery of the antinociceptive response to morphine; however, the morphine dose–response curve did not return to its original position by five weeks after 10 mg/kg clocinnamox. Whole brain membranes were prepared from separate groups of rats for determination of binding parameters of [^{3}H][D-Ala^2, *N*-Me-Phe^4,Gly^5-ol]-enkephalin (DAMGO). Clocinnamox dose-dependently decreased [^{3}H]DAMGO binding ex vivo and the decreased binding was a result of changes in B_{max}. The control B_{max} for [^{3}H]DAMGO was 234 ± 8 fmol/mg protein; in membranes prepared from rats pretreated with 10 mg/kg clocinnamox, the B_{max} value for [^{3}H]DAMGO was 54 ± 2 fmol/mg protein. The B_{max} values for [^{3}H]DAMGO binding after an injection of 10 mg/kg clocinnamox returned towards control values gradually, four weeks after clocinnamox the B_{max} was 178 ± 10 fmol/mg protein. These results suggest that clocinnamox is a long-lasting, nonequilibrium μ-opioid receptor antagonist in rats. © 1997 Elsevier Science B.V.

Keywords: Analgesia; Morphine; Clocinnamox; (Rat); [^{3}H]DAMGO ([^{3}H][D-Ala^2, *N*-Me-Phe^4,Gly^5-ol]-enkephalin); Opioid receptor binding

1. Introduction

Clocinnamox, a cinnamoylamino derivative of naltrexone, was first described as a long-lasting opioid antagonist with only very weak agonist effects in 1988 (Lewis et al., 1988). Clocinnamox has no antinociceptive effects as measured by increased tail-withdrawal latencies from warm water and is only partially effective in an acetic acid writhing test (Comer et al., 1992; Lewis et al., 1988). However, clocinnamox is an effective opioid antagonist and will attenuate the antinociceptive effects of several μ-opioid receptor agonists including morphine, fentanyl and etonitazene (Comer et al., 1992; Burke et al., 1994). The opioid antagonist effects of clocinnamox in mice are dose-dependent and of long duration. Low doses of clocinnamox will displace agonist dose–response curves to the right in a parallel fashion and high doses of clocinnamox will decrease the slope of the dose–response curves obtained with morphine-like drugs. Furthermore, a single injection of clocinnamox will antagonize the effects of morphine-like drugs for four to twelve days, the duration of the antagonism depending on the dose of clocinnamox that is administered (Burke et al., 1994).

Studies in monkeys indicate that the effects of clocinnamox in nonhuman primates are similar to clocinnamox effects reported in mice. In monkeys, a 3 h pretreatment with 0.1 mg/kg clocinnamox will displace the antinociceptive dose–effect curves of morphine and alfentanil to the right and will decrease the maximum effect obtained with these μ-opioid receptor agonists, however, clocinnamox does not antagonize the antinociceptive effects of the κ-opioid receptor agonist, U50-488H (Zernig et al., 1994). As in mice, the μ-opioid receptor antagonism pro-

* Corresponding author. Present address: Harvard Medical School, ADARC/McLean Hospital, 115 Mill Street, Belmont, MA 02178, USA. Tel.: (1-617) 855-2148; Fax: (1-617) 855-2417.

duced by clocinnamox in rhesus monkeys persists for two to three weeks. The ability of clocinnamox to antagonize μ-opioid receptor agonist effects in an insurmountable manner and the long duration of this antagonism are similar to the reported effects of the opioid receptor alkylating agents β-funaltrexamine (β-FNA) and β-chlornaltrexamine (Ward et al., 1982; Dykstra et al., 1987) and suggest that in vivo clocinnamox acts as an irreversible or nonequilibrium antagonist.

In binding studies, clocinnamox has been shown to have 20–30-fold selectivity for μ- over δ- and κ-opioid receptors and studies show that a dose of 3.2 mg/kg clocinnamox decreases the ex vivo binding of [^{3}H][D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]-enkephalin (DAMGO) to μ-opioid binding sites by approximately 88% without affecting the ex vivo binding of [^{3}H] *p*-Cl-[D-Pen^2,D-Pen^5]-enkephalin (DPDPE) or [^{3}H]bremazocine to δ- and κ-opioid binding sites (Lewis et al., 1988; Zernig et al., 1994). Clocinnamox decreases the B_{max} of [^{3}H]DAMGO in mouse whole brain membranes without affecting the K_D, suggesting that clocinnamox decreases the density of μ-opioid binding sites without altering receptor affinity (Burke et al., 1994; Chan et al., 1995). Inhibition of ex vivo [^{3}H]DAMGO binding by clocinnamox increases dose-dependently and high doses of clocinnamox, e.g., 32 mg/kg, virtually eliminate [^{3}H]DAMGO binding to mouse whole brain membranes. Similar to its in vivo antagonist actions, the inhibitory effects of clocinnamox on opioid binding to mouse brain membranes persist for eight to twelve days, depending on the dose of clocinnamox (Burke et al., 1994). In a recent study, in vitro binding by clocinnamox was shown to be wash-resistant, supporting the idea that clocinnamox binds μ-opioid receptors in an irreversible manner (Zernig et al., 1996). Overall, the effects of clocinnamox in opioid radioligand binding experiments are consistent with its characterization as a nonequilibrium antagonist.

There have been few reports of the effects of clocinnamox in rats. The original report on the effects of clocinnamox indicated that clocinnamox antagonizes the antinociceptive effects of morphine in a tail-withdrawal procedure in rats (Lewis et al., 1988). In other reports on the effects of clocinnamox in rats, a dose of 10 mg/kg clocinnamox displaced the morphine dose–response curve for discriminative stimulus effects approximately 10-fold to the right and, in a separate study, resulted in an approximately 10-fold shift to the right in the dose–effect function for antinociceptive effects of etonitazene (Walker et al., 1996; Walker, 1997). However, parametric studies of the in vivo effects of clocinnamox in rats have not been completed, nor have any studies to date examined the effects of clocinnamox on μ-opioid receptor binding to whole rat brain membranes. Therefore, the present experiments were undertaken to more fully characterize the in vivo and ex vivo effects of clocinnamox as a μ-opioid receptor antagonist in rats.

2. Materials and methods

2.1. Subjects

Female Sprague–Dawley rats, weighing 180–205 g at the start of the experiment were used. Subjects were group housed in either standard suspended wire cages or in standard polycarbonate cages. Vivaria were temperature-controlled and under 12 h light/dark cycles. Food (Purina Rodent Chow, Purina Mills, St. Louis, MO, USA) and water were available ad libitum.

2.2. Antinociception assay

Antinociception was assessed using a warm-water tail-withdrawal procedure. Rats were wrapped in a surgical towel and the distal third of the tail was immersed in water held at either 25° or 50°C; the different temperatures were presented in a random order within each test cycle. The 25° water served as a non-noxious control and data from these trials were not recorded. Latency to remove the tail from 50° water was recorded using a hand-held timer and a 25 s maximum was imposed to minimize damage to the tail. Test sessions began with two habituations trials at each temperature; the third trial was considered the baseline trial and was immediately followed by injection of the lowest dose of morphine. Complete dose–effect curves were generated in each rat using a cumulative dosing procedure similar to that described previously (Adams et al., 1990). Briefly, 25 min after each injection of morphine the rats were retested and then injected with the next dose of drug such that each injection increased the cumulative dose by 0.25 or 0.5 log units. This procedure continued until the rats failed to withdraw their tails from 50°C water within 25 s or until increasing doses failed to produce increases in tail-withdrawal latencies.

Rats were randomly assigned to one of five groups ($n = 6$) designated vehicle, 0.1-clocinnamox, 1.0-clocinnamox, 10a-clocinnamox and 10b-clocinnamox. Three to seven days after baseline morphine dose–effect curves were determined, rats received either vehicle or clocinnamox. Responses to morphine were redetermined at different times (1 h–5 weeks) after the clocinnamox injections.

2.3. Mu-opioid receptor binding

Rats were pretreated 1 h–4 weeks prior to killing with either clocinnamox (0.1–10.0 mg/kg) or vehicle. [^{3}H]DAMGO binding to whole rat brain membranes was determined according to a previously described method with minor modifications (Clark et al., 1988). Briefly, whole brain (minus cerebellum) was rapidly dissected out and minced in ice-cold Tris-buffer (50 mM Tris–HCl, pH 7.4 at 25°C, with 0.1 mM phenylmethylsulfonylfluoride). Tissues from groups of three rats were pooled together.

Following gross disruption with surgical scissors, the tissue was homogenized in 40 ml Tris by ten stokes in a Teflon glass homogenizer (Dounce; pestle clearance 60–90 μm). The homogenate was centrifuged at $40\,000 \times g$ for 15 min at 4°C. Homogenates were washed either one or three times by resuspension in Tris-buffer followed by centrifugation, after which pellets were resuspended in 5 volumes of Tris and stored at −70°C. Protein content was determined according to Bradford (1976) using a commercially available reagent (Bio-Rad; Richmond, CA, USA). Protein concentration of the membrane suspensions ranged from 378 to 588 μg/ml.

Binding assays were conducted in duplicate in 50 mM Tris-buffer. Membrane homogenates were incubated for 3 h at 25°C with 0.1–6.6 nM of [^{3}H]DAMGO (58 Ci/mmol; Amersham, Des Plaines, IL, USA) in the presence or absence of 1 μM unlabeled DAMGO dissolved in dimethyl sulfoxide (DMSO). Total assay volume was 0.5 ml and the final assay concentration of DMSO was 1%. DMSO up to a concentration of 5% does not inhibit [^{3}H]DAMGO binding (Zernig et al., 1996). The reaction was stopped by rapid filtration with a 24-sample cell harvester over glass-fiber, followed by three washings with approximately 5 ml of ice-cold buffer. Radioactivity remaining on the filters was determined by liquid scintillation counting. Because there were no differences in the binding parameters of [^{3}H]DAMGO among the different vehicle-treated preparations, data from these groups were combined to form a single control.

2.4. Data analysis

Tail-withdrawal latencies are expressed as a percentage of maximum possible effect (MPE) according to the equation: %MPE = ((test latency − baseline latency)/(25 s − baseline latency)) × 100%. When possible, individual ED_{50} values were calculated from linear regression of the dose–effect functions and means and 95% confidence intervals were obtained for each group of rats. Control tail-withdrawal latencies and baseline ED_{50} values were compared using a two-way analysis of variance (ANOVA). B_{max} and K_d values were determined by nonlinear regression using GraphPad Prism (GraphPad, San Diego, CA, USA).

2.5. Drugs

Morphine sulfate (Mallinckrodt, St. Louis, MO, USA) was dissolved in sterile water up to a concentration of 100 mg/ml. Clocinnamox (synthesized by Dr. John W. Lewis and colleagues, University of Bristol, Bristol, UK) was administered as a slurry in sterile water at concentrations of 0.1 and 1.0 mg/ml. Drugs were injected s.c. in volumes of 1–10 ml/kg. Morphine doses are expressed as the weight of the salt.

3. Results

3.1. Antinociception

Control tail-withdrawal latencies, listed in Table 1, did not differ significantly between groups prior to clocinnamox administration ($F(4,24) = 1.29$, $P > 0.2$). Clocinnamox did not affect control tail-withdrawal latencies in a consistent manner although a two-way ANOVA of control tail-withdrawal latencies revealed significant effects for dose ($F(3,19) = 7.73$, $P < 0.01$) and time ($F(2,38) = 8.73$, $P < 0.01$) as well as an interaction of main effects ($F(2,41) = 2.41$, $P < 0.05$). Post-hoc analysis indicated that the control latencies of group 10a-clocinnamox were significantly different from all other groups at 24 h and 7 days after clocinnamox. Morphine (1.0–18.0 mg/kg) dose-dependently increased tail-withdrawal latencies in all groups during baseline testing. Baseline ED_{50} values for each group ranged from 4.5 to 10.8 mg/kg. Despite a two-fold range in the mean ED_{50} values, the differences between groups during baseline testing were not statistically significant ($F(4,24) = 1.91$, $P > 0.1$).

The antinociceptive responses to morphine were not affected by 24 h or 7 day pretreatment with either vehicle or the lowest dose of clocinnamox (Fig. 1). A 24 h

Table 1
Control tail-withdrawal latencies [a,b] (in s) before and after clocinnamox

Group	Baseline	1 h	24 h	7 days	2 weeks	3 weeks	4 weeks	5 weeks
Vehicle	3.8 ± 0.3	— [c]	3.6 ± 0.5	3.8 ± 0.2	—	—	—	—
0.1 mg/kg clocinnamox	4.4 ± 0.3	—	3.0 ± 0.2	3.1 ± 0.4	—	—	—	—
1.0 mg/kg clocinnamox	5.3 ± 0.5	—	4.3 ± 0.4	2.5 ± 0.1	—	—	—	—
10 mg/kg clocinnamox	5.2 ± 0.8	—	5.2 ± 0.7 [d]	4.3 ± 0.3 [d]	—	—	—	—
10 mg/kg clocinnamox	4.7 ± 0.8	3.9 ± 0.6	—	—	5.5 ± 1.1	3.7 ± 0.5	4.2 ± 0.7	7.0 ± 0.9

[a] Control tail-withdrawal latencies were determined immediately prior to the first injection of morphine.
[b] Values given are group means ± S.E.
[c] Control latencies were not determined.
[d] Significantly different from vehicle, 0.1-clocinnamox and 1.0-clocinnamox groups.

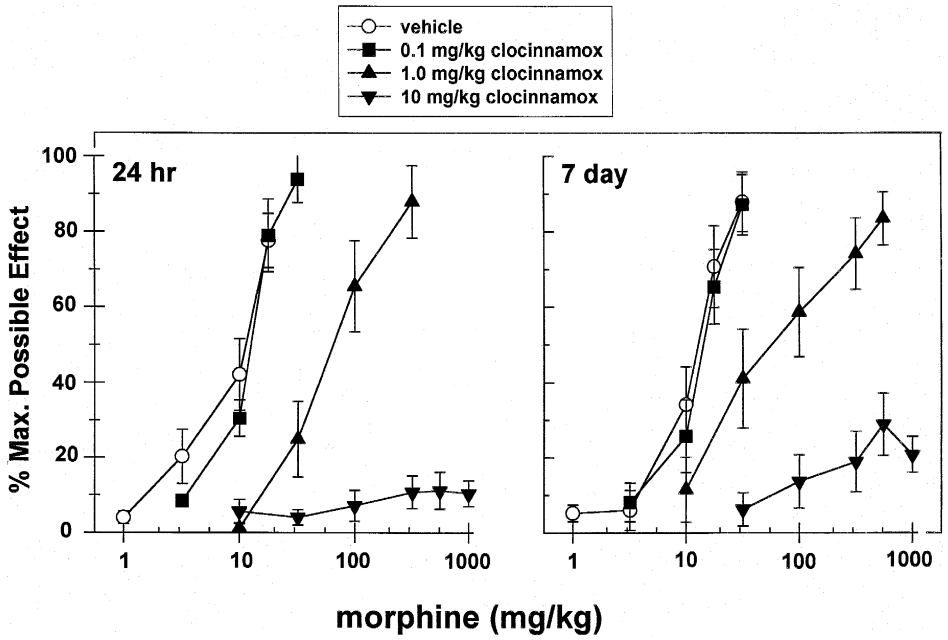

Fig. 1. Effects of different doses of clocinnamox on the antinociceptive responses to morphine at 24 h (left panel) and 7 days (right panel) after the clocinnamox injection. Abscissae: cumulative morphine dose in mg/kg. Ordinates: percentage of the maximum possible effect. Each point represents the group mean ($n = 6$) and vertical lines are ± 1 S.E.

pretreatment with 1.0 mg/kg clocinnamox displaced the morphine dose–effect function approximately one full log unit to the right of the dose–effect function of the vehicle-treated group; the morphine ED_{50} in the clocinnamox treated rats was 96 ± 1.6 mg/kg. Following pretreatment with the highest dose of clocinnamox, 10.0 mg/kg, doses of morphine up to 1000 mg/kg did not produce antinociceptive effects. The antagonism of morphine by 1 and 10 mg/kg clocinnamox lasted for at least 7 days (Fig. 1).

The time-course of the antagonism of morphine by 10 mg/kg clocinnamox was examined by determining morphine antinociceptive dose–effect functions in another group of rats (10b-clocinnamox) at 1 h and 2, 3, 4 and 5 weeks after the administration of 10 mg/kg clocinnamox. 1 h after the administration of clocinnamox, a cumulative dose of 100 mg/kg morphine produced approximately 50% of the maximum possible effect and doses of morphine greater than 100 mg/kg did not increase the antinociceptive response above 50% of the MPE (Fig. 2). As mentioned above, 1000 mg/kg morphine did not pro-

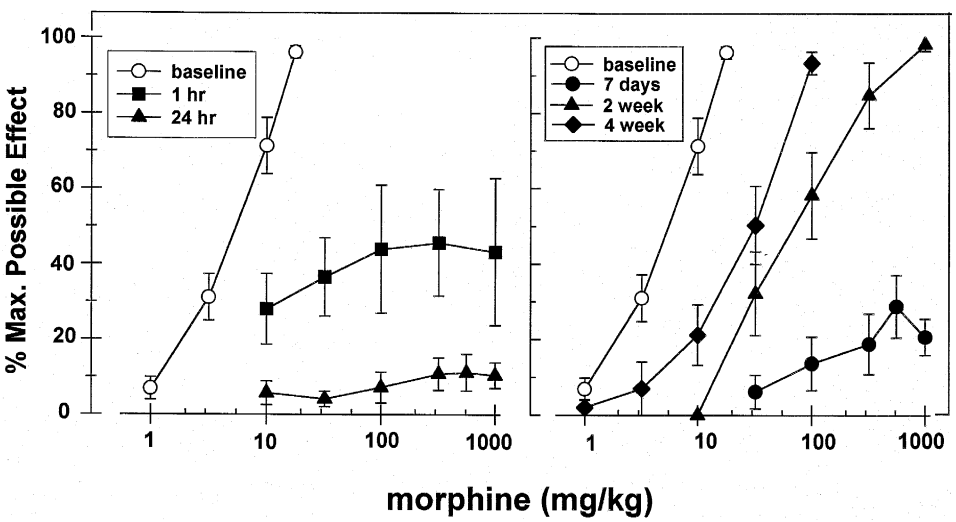

Fig. 2. Antinociceptive responses to morphine at different times after injection of 10 mg/kg clocinnamox; baseline curves were determined before the clocinnamox injection. Abscissae: cumulative morphine dose in mg/kg. Ordinates: percentage of the maximum possible effect. Each point represents the group mean ($n = 6$) and vertical lines are ± 1 S.E. Data for the curves at 24 h and 7 days after clocinnamox are redrawn from Fig. 1.

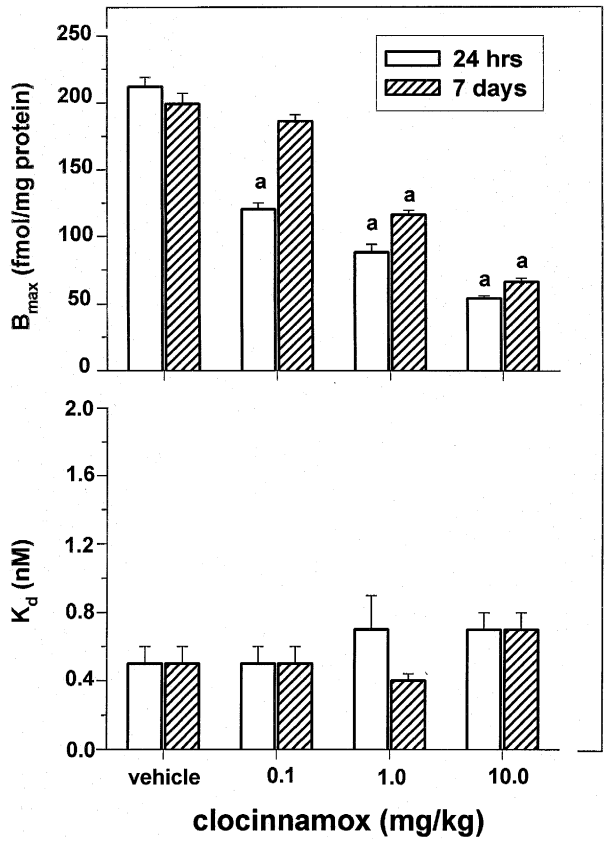

Fig. 3. B_{max} values (top panel) and K_d values (bottom panel) for [^{3}H]DAMGO at 24 h (open bars) and 7 days (hatched bars) after different doses of clocinnamox. Abscissae: clocinnamox dose in mg/kg. Ordinates: B_{max} value in fmol/mg protein (top) and K_d value in nmol (bottom). Vertical lines represent +1 S.E. ([a] different from vehicle; $P < 0.05$).

duce greater than 30% antinociception in rats at either 24 h or 7 days after injection of 10 mg/kg clocinnamox. 2 weeks after the clocinnamox injection, morphine was again able to produce 100% of the maximum possible antinociceptive effect, however, the morphine dose–effect function was displaced more than one log unit to the right of the baseline morphine dose–effect function. The morphine ED_{50} 2 weeks after a single injection of 10 mg/kg clocinnamox was 80 ± 1.4 mg/kg. At 3 to 5 weeks after clocinnamox, the morphine dose–effect function was shifted back toward the baseline curve, however, the baseline antinociceptive effects of morphine were not recaptured. 5 weeks after the administration of 10 mg/kg clocinnamox the ED_{50} value of morphine for producing antinociception was 23 ± 1.2 mg/kg, more than 5-fold higher than the baseline morphine ED_{50} value.

3.2. Ex vivo saturation binding

In membranes prepared from rats killed either 24 h or 7 days after a vehicle injection, the B_{max} values of [^{3}H]DAMGO were 212 ± 7 and 199 ± 8 fmol/mg protein, respectively; the K_d was 0.5 ± 0.1 nM in both groups. Clocinnamox administered 24 h prior to killing dose-dependently decreased B_{max} values for [^{3}H]DAMGO. The lowest clocinnamox dose decreased the B_{max} of [^{3}H]DAMGO to roughly 55% of control values; the highest dose of clocinnamox decreased the B_{max} to approximately 25% of control values (Fig. 3). B_{max} values were greater at 7 days after the clocinnamox injections than at 24 h after clocinnamox and the degree of recovery was related to the dose of clocinnamox administered and/or the magnitude of the initial decrease of B_{max}. For example, 24 h after the administration of 0.1 mg/kg clocinnamox the B_{max} of [^{3}H]DAMGO was 120 fmol/mg protein and by 7 days the B_{max} had recovered to 186 fmol/mg protein. In contrast, 24 h after 10 mg/kg clocinnamox the B_{max} of [^{3}H]DAMGO was 55 fmol/mg protein and by 7 days after clocinnamox this value increased only to 66 fmol/mg protein. Clocinnamox did not affect the affinity of the binding sites for [^{3}H]DAMGO at either 24 h or 7 days; K_d values for [^{3}H]DAMGO in membranes prepared from clocinnamox-treated rats were similar to those obtained in control membranes (Fig. 3).

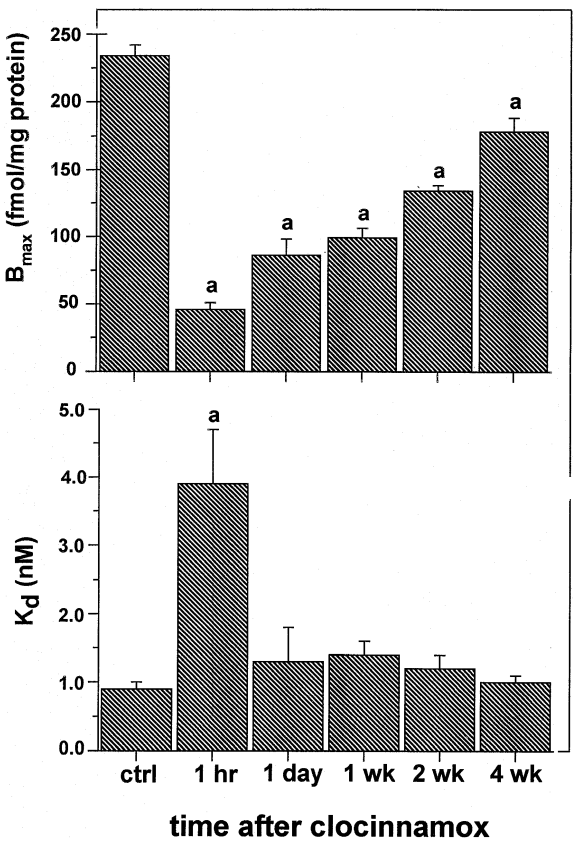

Fig. 4. B_{max} values (top panel) and K_d values (bottom panel) for [^{3}H]DAMGO at different times after 10 mg/kg clocinnamox; control data are from vehicle-treated preparations. Abscissae: time since clocinnamox injection. Ordinates: B_{max} value in fmol/mg protein (top) and K_d value in nmol (bottom). Vertical lines represent +1 S.E. ([a] different from control; $P < 0.05$).

Table 2
B_{max} and K_d values (with 95% C.L.) for [^{3}H]DAMGO in control [a] and 10 mg/kg clocinnamox-treated [b] membrane preparations

	Control		1 h clocinnamox		24 h clocinnamox	
	1 wash	3 washes	1 wash	3 washes	1 wash	3 washes
B_{max} (fmol/mg protein)	224 (204, 244)	276 [c] (246, 306)	46 (33, 59)	37 (22, 51)	86 (54, 118)	97 (77, 118)
K_d (nM)	0.8 (0.6, 1.0)	0.7 (0.5, 1.0)	3.9 (1.8, 6.1)	2.7 (0.3, 5.0)	1.3 (0.1, 2.7)	1.5 (0.7, 2.4)

[a] Control animals were killed 1 h after a vehicle injection.
[b] Clocinnamox-treated animals were killed 1 or 24 h after clocinnamox administration.
[c] Different from control preparations washed one time ($P < 0.05$).

The time-course of the effects of 10 mg/kg clocinnamox on [^{3}H]DAMGO binding was examined at 1 and 24 h and at 7, 14 and 28 days after injection of 10 mg/kg clocinnamox. The effects of 10 mg/kg clocinnamox on ex vivo binding were greatest 1 h after clocinnamox administration. The B_{max} of [^{3}H]DAMGO was 20% of control values and the K_d of [^{3}H]DAMGO was increased to almost 4 nM at 1 h after 10 mg/kg clocinnamox (Fig. 4). Onset of the recovery of [^{3}H]DAMGO binding was apparent by 24 h after the clocinnamox injection and the number of binding sites steadily increased throughout the four week period. However, control B_{max} values of [^{3}H]DAMGO binding were not recovered within 28 days after the administration of 10 mg/kg clocinnamox. In contrast, the K_d values of [^{3}H]DAMGO were within control values by 24 h after clocinnamox administration.

Membrane preparations from rats that received 10 mg/kg clocinnamox either 1 or 24 h prior to killing were washed three times, in order to determine whether residual clocinnamox remained in the preparations. B_{max} values for [^{3}H]DAMGO were slightly higher in control preparations washed three times compared to those washed only once. There were no differences in the binding parameters of [^{3}H]DAMGO in clocinnamox-treated preparations washed three times compared to those washed only once (Table 2).

4. Discussion

In the present study, a low dose of clocinnamox displaced the dose–effect curve for morphine antinociception to the right and a high dose of clocinnamox insurmountably antagonized morphine. The effects of clocinnamox on ex vivo binding paralleled its in vivo antagonist effects in that clocinnamox decreased the B_{max} values of [^{3}H]DAMGO in rat whole brain membranes in a dose-dependent manner. The inhibition of binding at the μ-opioid site and the antagonism of the antinociceptive effects of morphine persisted for at least four weeks after injection of a single dose of 10 mg/kg clocinnamox. Previous reports on the effects of clocinnamox demonstrated that clocinnamox dose-dependently antagonizes the antinociceptive effects of μ-opioid agonists in rodents (Burke et al., 1994; Walker, 1997) and that these effects in mice are accompanied by decreases in μ-opioid binding to whole brain membranes (Burke et al., 1994). The present experiments extend these results and are consistent with the characterization of clocinnamox as a nonequilibrium μ-opioid receptor antagonist in rats.

The ability to decrease the maximum agonist response functionally distinguishes the effects of noncompetitive and irreversible antagonists from the surmountable effects of competitive antagonists. Results obtained from in vitro binding assays strongly suggest that the flattening of in vivo opioid agonist dose–effect curves in the presence of irreversible antagonists is due to the elimination of receptors. For example, incubation of membrane preparations with 5–1000 nM of the μ-opioid selective alkylating agent β-funaltrexamine, has been shown to decrease the B_{max} values of [^{3}H]DAMGO, [^{3}H]morphine and [^{3}H]naltrexone (Ward et al., 1985; Tam and Liu-Chen, 1986), whereas intracerebroventricular (i.c.v.) doses of 10–20 nmol β-funaltrexamine were found to decrease the maximum antinociceptive effects of morphine and other μ-opioid receptor agonists (Adams et al., 1990). Similarly, the more recently synthesized opioid antagonists, *N*-cyclopropylmethylnor-5β-methyl-14β-(*p*-nitrocinnamoylamino)-7,8-dihydromorphinone (*N*-CPM-MET-CAMO) and clocinnamox, have been shown to decrease in vitro binding of [^{3}H]DAMGO to membrane preparations (Jiang et al., 1994; Zernig et al., 1996) and to decrease the maximum antinociceptive effect produced by μ-opioid receptor agonists in mice (Comer et al., 1992; Jiang et al., 1994). Thus, it seems reasonable to assume that the in vivo antagonist effects of nonequilibrium μ-opioid antagonists result from a decrease in available μ-opioid receptors and, in turn, a decreased number of [^{3}H]DAMGO binding sites.

Although clocinnamox dose-dependently decreased B_{max} values of [^{3}H]DAMGO in the present study, the lowest dose of clocinnamox, 0.1 mg/kg, did not affect the dose–effect curve for morphine antinociception. It is unclear how a reduction of μ-opioid binding sites by almost 50% did not result in a decreased potency of morphine in producing antinociceptive effects in the warm-water withdrawal procedure. It is equally difficult to explain how an equivalent 50% reduction of [^{3}H]DAMGO binding seven days after administration of a higher dose of clocinnamox, 1 mg/kg, did result in an antagonism of the antinociceptive effects of morphine. It is worth noting that often there is also a discordance between the in vivo antagonist effects

of the alkylating agent β-funaltrexamine and its effect on opioid radioligand binding ex vivo. However, the differences between the in vivo and the ex vivo effects of β-funaltrexamine tend to be opposite to the results obtained with clocinnamox in the present studies. For example, doses of β-funaltrexamine that have either no effect on ex vivo binding of [^{3}H]DAMGO or that, under optimal conditions, decrease the binding of [^{3}H]DAMGO by 40–50% will completely antagonize the antinociceptive effects of morphine in rats (Adams et al., 1987; Liu-Chen et al., 1991; Liu-Chen et al., 1995). μ-Opioid receptors are widely distributed within the CNS (Mansour et al., 1995) and it is likely that not all μ-opioid receptors revealed by [^{3}H]DAMGO binding sites necessarily mediate the antinociceptive effects of morphine. With this in mind, an exact correspondence between the in vivo and ex vivo effects of clocinnamox was not expected. Nonetheless, the mechanism underlying the disparate effects of β-funaltrexamine and clocinnamox on ex vivo [^{3}H]DAMGO binding and in vivo antagonism of morphine remain unresolved.

Although both β-funaltrexamine and clocinnamox have long-lasting effects on ex vivo [^{3}H]DAMGO binding, their interaction with μ-opioid binding sites may differ. β-funaltrexamine initially binds μ-opioid receptors competitively and, after the initial period of competition, β-funaltrexamine appears to covalently bind μ-opioid binding sites (Liu-Chen and Phillips, 1987). Clocinnamox also appears to initially interact with μ-opioid binding sites in a competitive manner. In the current study, for example, the increased K_D of [^{3}H]DAMGO at 1 h after clocinnamox in the binding assay and the partially surmountable effects of 10 mg/kg clocinnamox by morphine in vivo suggest that [^{3}H]DAMGO and morphine effectively compete with clocinnamox at μ-opioid receptors during the 1 h after clocinnamox injection. At times later than 1 h after clocinnamox, however, the K_D of [^{3}H]DAMGO was similar to control values and morphine was unable to elicit an antinociceptive response suggesting that, like β-funaltrexamine, clocinnamox binds μ-opioid receptors in a noncompetitive fashion. However, the precise mechanisms by which clocinnamox produces its nonequilibrium effects are not known. Despite the presence of a cinnamoylamino substituent which can serve as a Michael acceptor, there is no evidence that clocinnamox actually alkylates μ-opioid receptors. In fact, although clocinnamox is reportedly resistant to washout (Zernig et al., 1996; Aceto et al., 1989) one study with a tritiated form of clocinnamox reported that in vitro [^{3}H]clocinnamox is a fully reversible radioligand, albeit one with slow dissociation kinetics (Zernig et al., 1995). Therefore, it is unclear whether the nonequilibrium antagonism produced by clocinnamox is due to receptor inactivation or a slow rate of dissociation from the receptor.

Perhaps the most surprising result of the present experiments is the long duration of action of clocinnamox. In both binding and behavioral studies, recovery to control values was not seen within 4 weeks after the injection of the highest dose of clocinnamox. Such a long duration of opioid antagonism is not unique to clocinnamox. The κ-opioid receptor antagonist norbinaltorphimine may antagonize the behavioral effects of κ-opioid receptor agonists for weeks in mice and rats (Horan et al., 1992; Jones and Holtzman, 1992) and for months in pigeons (Jewett and Woods, 1995). Furthermore, suitable doses of β-funaltrexamine may inhibit [^{3}H]DAMGO binding for over 18 days (Martin et al., 1995). It is interesting to consider that the duration of action of these drugs appears to be longer than proposed opioid receptor turnover rates of 3 to 6 days (Standifer et al., 1994; Zernig et al., 1994). This suggests that even days after administration clocinnamox is able to bind to newly synthesized receptors, indicating that, in addition to possible receptor inactivation, the long-lasting antagonism produced by clocinnamox also may be due to pharmacokinetic factors, such as slow clearance or tissue sequestration of the drug.

In conclusion, clocinnamox is an effective, long-lasting antagonist of morphine in rats. The results of binding studies suggest that clocinnamox acts primarily by decreasing the number of available μ-opioid receptors. However, the mechanisms by which clocinnamox reduces receptor number are not known and it is possible that other factors, such as a slow dissociation rate or slow clearance, also contribute to the very long duration of action of clocinnamox.

Acknowledgements

This work was supported by PHS Grants DA 00254, DA 07268, DA 05676, MH 45232, and MH 00427. We thank Dr. Elizabeth A. Young and Dr. Patricia B. Lieberman for their assistance in completing these studies and Dr. John Lewis for generously providing clocinnamox. We also thank Dr. Jack Bergman and Dr. John Traynor for comments on the manuscript.

References

Aceto, M.D., Bowman, E.R., May, E.L., Harris, L.S., Woods, J.H., Smith, C.B., Medzihradsky, F., Jacobson, A.E., 1989. Very long-lasting narcotic antagonists: The 14β-*p*-substituted cinnamoylaminomorphinones and their partial *mu* agonist codeinone relatives. Arzneim. Forsch. Drug Res. 39, 570–575.

Adams, J.U., Andrews, J.S., Hiller, J.M., Simon, E.J., Holtzman, S.G., 1987. Effects of stress and β-funaltrexamine on morphine analgesia and opioid binding in rats. Life Sci. 41, 2835–2844.

Adams, J.U., Paronis, C.A., Holtzman, S.G., 1990. Assessment of relative intrinsic activity of mu-opioid analgesics in vivo by using β-funaltrexamine. J. Pharmacol. Exp. Ther. 255, 1027–1032.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Burke, T.F., Woods, J.H., Lewis, J.W., Medzihradsky, F., 1994. Irreversible opioid antagonist effects of clocinnamox on opioid analgesia and mu receptor binding in mice. J. Pharmacol. Exp. Ther. 271, 715–721.

Chan, K., Brodsky, M., Davis, T., Franklin, S., Inturrisi, C.E., Yoburn, B.C., 1995. The effects of the irreversible mu-opioid receptor antagonist clocinnamox on morphine potency, receptor binding and receptor mRNA. Eur. J. Pharmacol. 287, 135–143.

Clark, M.J., Carter, B.D., Medzihradsky, F., 1988. Selectivity of ligand binding to opioid receptors in brain membranes from the rat, monkey and guinea pig. Eur. J. Pharmacol. 148, 343–351.

Comer, S.D., Burke, T.F., Lewis, J.W., Woods, J.H., 1992. Clocinnamox: A novel, systemically-active irreversible opioid antagonist. J. Pharmacol. Exp. Ther. 262, 1051–1056.

Dykstra, L.A., Gmerek, D.E., Winger, G., Woods, J.H., 1987. Kappa opioids in rhesus monkeys. II. Analysis of the antagonistic actions of quadazocine and β-funaltrexamine. J. Pharmacol. Exp. Ther. 242, 421–427.

Horan, P., Taylor, J., Yamamura, H.I., Porreca, F., 1992. Extremely long-lasting antagonist actions of nor-binaltorphimine (nor-BNI) in the mouse tail-flick test. J. Pharmacol. Exp. Ther. 260, 1237–1243.

Jewett, D.J., Woods, J.H., 1995. Nor-binaltorphimine: An ultra-long acting kappa-opioid antagonist in pigeons. Behav. Pharm. 6, 815–821.

Jiang, Q., Sebastian, A., Archer, S., Bidlack, J.M., 1994. 5β-methyl-14β-(*p*-nitrocinnamoylamino)-7,8-dihydomorphinone and its corresponding *n*-cyclopropylmethyl analog, *n*-cyclopropylmethylnor-5β-methyl-14β-(*p*-nitrocinnamoylamino)-7,8-dihydomorphinone: Mu-selective irreversible opioid antagonists. J. Pharmacol. Exp. Ther. 268, 1107–1113.

Jones, D.N.C., Holtzman, S.G., 1992. Long-term *k*-opioid receptor blockade following nor-binaltorphimine. Eur. J. Pharmacol. 215, 345–348.

Lewis, J., Smith, C., McCarthy, P., Walter, D., Kobylecki, R., Myers, M., Haynes, A., Lewis, C., Waltham, K., 1988. New 14-aminomorphinones and codeinones. NIDA Res. Monogr. 90, 136–143.

Liu-Chen, L.-Y., Phillips, C.A., 1987. Covalent labeling of mu opioid binding site by [^{3}H] β-funaltrexamine. Mol. Pharmacol. 32, 321–329.

Liu-Chen, L.-Y., Li, S., Wheeler-Aceto, H., Cowan, A., 1991. Effects of intracerebroventricular β-funaltrexamine on mu and delta opioid receptors in the rat: Dichotomy between binding and antinociception. Eur. J. Pharmacol. 203, 195–202.

Liu-Chen, L.-Y., Yang, H.-H., Li, S., Adams, J.U., 1995. Effect of intracerebroventricular β-funaltrexamine on *mu* opioid receptors in the rat brain: Consideration of binding condition. J. Pharmacol. Exp. Ther. 273, 1047–1056.

Mansour, A., Fox, C.A., Akil, H., Watson, S.J., 1995. Opioid-receptor mRNA expression in the rat CNS: Anatomical and functional implications. Trends Neurosci. 18, 22–29.

Martin, T.J., Dworkin, S.I., Smith, J.E., 1995. Alkylation of mu opioid receptors by β-funaltrexamine in vivo: Comparison of the effects on in situ binding and heroin self-administration in rats. J. Pharmacol. Exp. Ther. 272, 1131–1140.

Standifer, K.M., Chien, C.-C., Wahlestedt, C., Brown, G.P., Pasternak, G.W., 1994. Selective loss of delta opioid analgesia and binding by antisense oligodeoxynucleotides to a delta opioid receptor. Neuron 12, 805–810.

Tam, S.W., Liu-Chen, L.-Y., 1986. Reversible and irreversible binding of β-funaltrexamine to mu, delta and kappa opioid receptors in guinea pig brain membranes. J. Pharmacol. Exp. Ther. 239, 351–357.

Walker, E.A., 1997. Etonitazene analgesia in rats: Antagonism, tolerance and cross-tolerance. NIDA Res. Monogr. 174, 73.

Walker, E.A., Richardson, T.M., Young, A.M., 1996. In vivo apparent pA_2 analysis in rats treated with either clocinnamox or morphine. Psychopharmacology 125, 113–119.

Ward, S.J., Portoghese, P.S., Takemori, A.E., 1982. Pharmacological profiles of β-funaltrexamine (β-FNA) and β-chlornaltrexamine (β-CNA) on the mouse vas deferens preparation. Eur. J. Pharmacol. 80, 377–384.

Ward, S.J., Fries, D.S., Larson, D.L., Portoghese, P.S., Takemori, A.E., 1985. Opioid receptor binding characteristics of the non-equilibrium mu-antagonist, β-funaltrexamine (β-FNA). Eur. J. Pharmacol. 107, 323–330.

Zernig, G., van Bemmel, B.G., Lewis, J.W., Woods, J.H., 1995. Characterization of [^{3}H]clocinnamox binding in mouse brain membranes. Analgesia 1, 874–877.

Zernig, G., Burke, T., Lewis, J.W., Woods, J.H., 1996. Mechanism of clocinnamox blockade of opioid receptors: Evidence from in vitro and ex vivo binding and behavioral assays. J. Pharmacol. Exp. Ther. 279, 23–31.

Zernig, G., Butelman, E.R., Lewis, J.W., Walker, E.A., Woods, J.H., 1994. In vivo determination of mu opioid receptor turnover in rhesus monkeys after irreversible blockade with clocinnamox. J. Pharmacol. Exp. Ther. 269, 57–65.