

CLOCINNAMOX BLOCKS ONLY MU RECEPTORS IRREVERSIBLY: BINDING EVIDENCE

Zernig G*, Burke T,* Lewis JW**, Woods JH*,#, Departments of Pharmacology* and Psychology#, University of Michigan, Ann Arbor, MI 48109, USA and Department of Chemistry**, University of Bristol, Bristol, England

In rhesus monkey antinociception experiments, the cinnamoylaminomorphinone opioid antagonist clocinnamox at a dose of 0.1 mg/kg irreversibly inactivated 88% of the mu opioid receptor population (as calculated by *in vivo* Furchgott analysis) whereas kappa receptors remained unaffected (1). In contrast, *in vitro* radioligand binding assays showed only a moderate mu : delta : kappa - selectivity of clocinnamox (30 : 1.5 : 1; ref. 2). This apparent discrepancy was investigated in mice using both *in vitro* and *ex vivo* opioid binding studies.

Opioid receptor binding was determined using [³H]DAMGO for mu-, [³H]-p-CI-DPDPE for delta-, and [³H]bremazocine in presence of 1 μM DAMGO and 1 μM DPDPE for kappa opioid receptors in whole mouse brain membranes (buffer: 50 mM Tris-HCl pH 7.4, 0.1 mM phenylmethylsulfonyl fluoride, 1% dimethyl sulfoxide; 2 h incubation at 25°C). Clocinnamox showed pseudo-K_i and K_i values of 0.25 nM for mu receptors (pK_i, 8.41 ± 0.07; Hill slope, 1.0 ± 0.1; means ± S.E.), 1.6 nM for delta receptors (pK_i, 8.09 ± 0.17; Hill slope, 0.9 ± 0.2), and 7.2 nM for kappa receptors (pK_i, 7.80 ± 0.9; Hill slope, 0.1 ± 0.1), corresponding to a 29 : 5 : 1-selectivity for mu : delta : kappa, thus confirming previous studies. Similarly, clocinnamox' agonistic codeinone derivative, methoclocinnamox (3), yielded pseudo-K_i and K_i values of 0.46 nM for mu receptors (pK_i, 9.34 ± 0.16; Hill slope, 1.1 ± 0.1), 4.5 nM for delta receptors (pK_i, 8.35 ± 0.21; Hill slope, 1.2 ± 0.1), and 29 nM for kappa receptors (pK_i, 7.54 ± 0.12; Hill slope, 1.1 ± 0.3), corresponding to a 63 : 6 : 1-selectivity for mu : delta : kappa. Thus, the present data are in accordance with previously published reports from other laboratories.

In a washout experiment, naltrexone (1 μM), clocinnamox and methoclocinnamox (10 nM and 1 μM each) were added to whole mouse brain membranes, incubated for 2 hours (i.e., binding competition experiment condition), then repeatedly washed with 150 volumes/wet weight fresh medium (3) and further incubated for up to 8 h at 25°C to allow for ligand dissociation. Aliquots of these membranes were tested for mu-, delta-, and kappa-opioid receptor binding at the end of the competition binding incubation period, and after 2 h (2 washes) and 8 h (4 washes). Neither the binding inhibition mediated by 10 nM clocinnamox nor that of 1 μM clocinnamox could be reversed from mu receptors even after 8h washing (e.g., 1 μM, 99.8% → 94%), whereas it was at least partially reversible at delta (99.7% → 69%) and kappa (86% → 62%) receptors. The binding inhibition mediated by methoclocinnamox, however, was at least partially reversible at all three opioid receptor subtypes. Naltrexone-mediated binding inhibition was completely reversed at the first washout period tested, i.e. 2 h (2 washes). In summary, the *in vitro* binding data indicate that although clocinnamox interacts with all three opioid receptor subtypes, only its interaction with the mu receptor is of an irreversible nature.

Similar results were obtained in *ex vivo* binding experiments. Mice were treated with 1 - 10 mg/kg clocinnamox i.p., sacrificed 1 h to 28 d later, their brains homogenized and opioid receptor binding measured. Only *ex vivo* binding to mu receptors was inhibited by clocinnamox, delta and kappa receptor binding remained unaffected even after a clocinnamox pretreatment time of only 1 h. The [³H]DAMGO binding inhibition after clocinnamox was due to a decrease in receptor density whereas the affinity of the radioligand for the mu receptors remained essentially unchanged as already demonstrated in a previous study (5). After the acute and dose-dependent decrease of the mu receptor population by clocinnamox (1 h pretreatment; 1 mg/kg, 44 ± 3 % inhibition; 3.2 mg/kg, 90 ± 1 %; 10 mg/kg, 90 ± 2 %), *ex vivo* [³H]DAMGO binding reappeared with a half-lives of 4.2 d (after 1 mg/kg clocinnamox), 2.7 d (3.2 mg/kg), and 2.8 d (10 mg/kg).

The reappearance of mu opioid receptors was partially blocked by the protein synthesis inhibitor cycloheximide. Following previously published experimental protocols (6, 7), a loading dose of 50 mg/kg cycloheximide and a subsequent administration of 5 mg/(kg.h) cycloheximide by implanted osmotic minipumps was used to inhibit protein synthesis. Cycloheximide alone did not significantly change *ex vivo* [³H]DAMGO binding over 48 h (inhibition < 7%), after which the experiment had to be terminated because of the considerable side effects of cycloheximide. Administration of 3.2 mg/kg clocinnamox alone acutely decreased [³H]DAMGO binding to 12 ± 2 % of control. Binding partially recovered within the 48 h time period, exactly as expected from the previous time course experiments. In contrast, *ex vivo* [³H]DAMGO binding to brain membranes of sham-operated mice (implantation of empty minipumps) did not change over the whole time course. Finally, when mice were given both clocinnamox and cycloheximide, mu receptors acutely were decreased to about the same amount (i.e., 9 ± 2 % of the control population) as in the mice treated with clocinnamox alone. The reappearance of [³H]DAMGO binding, however, was decreased in comparison to the mice treated with clocinnamox alone: After 24 h, [³H]DAMGO binding after clocinnamox alone had returned to 49 ± 6 % of control, whereas it was 26 ± 1 % after combined administration of clocinnamox and cycloheximide. The respective values after 48 h were 62 ± 4 % for clocinnamox alone and 34 ± 5 % for the clocinnamox-cycloheximide combination. *Ex vivo* delta- and kappa opioid receptor binding remained unaffected by either clocinnamox or cycloheximide alone or their combination.

We also compared the time course of *ex vivo* [³H]DAMGO binding recovery after 3.2 mg/kg clocinnamox with estimates of q, the fraction of available receptors, as determined by Furchgott analysis of data obtained in warm-water tail withdrawal experiments. To that end, published time course experiments (Fig. 5 of ref.8) using morphine as an antagonist were repeated by different experimenters in our laboratory and extended to a time span of 16 days. Data were pooled and analyzed as detailed previously (1) to obtain q, the fraction of available receptors according to Furchgott (9) and Black and Leff (10). 3.2 mg/kg clocinnamox acutely depressed the q value to 0.3 (1 being the value for the control population). Q values returned with a half-life of 2.0 d, which was close to that of 2.7 d obtained when analyzing recovery of *ex vivo* [³H]DAMGO binding. Thus, Furchgott analysis of behavioral data again seemed to provide estimates of receptor population changes of reasonably high confidence.

Supported by Austrian Science Foundation Grants J0697-MED and J0882-MED, a NIDA INVEST fellowship (G.Z.), and USPHS Grant DA 00254.

REFERENCES

1. G.Zernig et al. (1994) J. Pharmacol. Exp. Ther. 268, 57-65
2. J.W. Lewis et al. (1988) NIDA Res. Monogr. 90,136-143
3. J.H. Woods et al. (1994) NIDA Res. Monogr., submitted
4. N. Johnson and G.W. Pasternak (1984) Mol. Pharmacol. 26,477-483
5. T. Burke et al. (1994) J. Pharmacol. Exp. Ther., in press
6. E.E.Quinton and N.R. Kramarcy (1977) Brain Res. 131,184-190
7. G. Sperk et al. (1978) Brain Res. 159,183-194
8. S. D. Comer et al. (1992) J. Pharmacol. Exp. Ther. 262, 1051-1056
9. R. F. Furchgott, Adv. Drug Res. 3, 21-56 (1966)
10. J.W. Black, P. Leff (1983) Proc. R. Soc. Lond. B 220,141-162