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Stereoselective μ- and δ-opioid receptor-related antinociception and binding with (+)-thebaine

Mario D. Aceto [a,*], Louis S. Harris [a], Mary E. Abood [a], Kenner C. Rice [b]

[a] *Department of Pharmacology and Toxicology, School of Medicine, Virginia Commonwealth University, Richmond, VA 23298-0613, USA*
[b] *Laboratory of Medicinal Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892, USA*

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Abstract

In vivo and in vitro binding studies with natural thebaine and its enantiomer, (+)-thebaine were conducted to elucidate further their interactions with the opioid system. (−)-Thebaine a key intermediate in the biosynthesis of morphine in the poppy plant (*Papaver somniferum*) and in mammalian tissue, was poorly effective antinociceptively in mice at doses to 30 mg/kg. Its principal behavioral manifestation was lethal convulsions. Naltrindole, at doses of 1 and 10 mg/kg did not block either the convulsions or lethal effects, suggesting that the δ-opioid receptor system was not involved in this action. Surprisingly, the dextrorotatory isomer exhibited significant antinociceptive activity in the tail-flick [ED_{50} = 8.9 (3.4–22.1) mg/kg], hot-plate [ED_{50} = 22.9 (10.9–48.1) mg/kg] and phenylquinone [ED_{50} = 1.9 (1.6–9.5) mg/kg] assays. Studies with opioid receptor-subtype antagonists, β-funaltrexamine, nor-binaltorphimine and naltrindole, indicated that antinociception was associated with μ- and δ-opioid receptors. Results of displacement experiments supported the in vivo data. Significant competition for [^{3}H]diprenorphine binding with both isomers for cloned μ- and δ-opioid receptors was observed. However, (−)-thebaine was more effective at the δ-opioid receptor ($K_i = 1.02 \pm 0.01$ μM) whereas (+)-thebaine was more effective at the μ-opioid receptor ($K_i = 2.75 \pm 0.01$ μM). Opioid-induced antinociception associated with unnatural thebaine raises the possibility of additional μ- and δ-opioid receptor sites.

Keywords: (−)-Thebaine; (+)-Thebaine; Stereoselectivity; Antinociception; μ-Opioid receptor; δ-Opioid receptor; (Mouse)

1. Introduction

Natural thebaine, the levorotatory isomer, is a key intermediate in the synthesis of morphine in the poppy plant, *Papaver somniferum*, (Barton et al., 1965; Battersby et al., 1965), and in the mammalian brain (Donnerer et al., 1986; Kodaira and Spector, 1988; Kodaira et al., 1989). This important precursor was reported to be practically devoid of analgesic properties and produced convulsions in mice (Aceto et al., 1977; Tortella et al., 1984) and it did not substitute for morphine in dependent rhesus monkeys (Aceto et al., 1977; Swain et al., 1977). We also reported severe convulsions in one monkey receiving 6 mg/kg s.c. which were terminated using pentobarbital. Preliminary results in our laboratory with limited quantities of the dextrorotatory isomer were surprising. Contrary to the prevailing dogma, this enantiomer displayed significant antinociceptive properties in the mouse sans convulsions and lethality. Interestingly, it did not substitute for morphine in morphine-dependent rhesus monkeys (Woods et al., 1985).

With the recent availability of additional supplies, we belatedly were able to characterize (+)-thebaine further and to compare the antipodes directly for their activity in vivo and in vitro.

2. Methods

2.1. In vivo studies

All animals received care according to 'Guide for the Care and Use of Laboratory Animals,' US Department of Health and Human Services, 1985. The facilities are certi-

* Corresponding author. Tel.: +1-804-828-8397; Fax: +1-804-828-2117; E-mail: maceto@hsc.vcu.edu

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fied by the American Association for the Accreditation of Laboratory Animal Care. These studies were approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University.

2.1.1. General methods

ICR male mice (Harlan–Sprague–Dawley, Indianapolis, IN) weighing 20–30 g, were used. Each animal was tested once only. All drugs except β-funaltrexamine were given subcutaneously (s.c.) in a volume of 0.1 ml/10 g of distilled water. β-Funaltrexamine was administered by the intracerebroventricular (i.c.v.) route in a volume of 5 μl/brain of sterile distilled water. (+)-Thebaine was first dissolved in dilute HCl. At least three doses were tested, and 6–10 animals per dose were used.

2.1.2. Tail-flick agonist or antagonist tests

This procedure was first reported by D'Amour and Smith (1941) and modified by us (Dewey et al., 1970; Dewey and Harris, 1971). Briefly, the mouse's tail was placed in a groove which contained a slit under which was located a photoelectric cell. When the heat source or noxious stimulus was turned on, heat was focused on the tail, and the mouse responded by flicking its tail out of the groove. As a result, light passed through the slit and activated a photocell which, in turn, stopped the recording timer. The heat source was adjusted to produce tail-flick latencies of 2–4 s under control conditions. Only mice meeting this criterion were used. Mice were injected with test drug or vehicle and tested 20 min later. Antinociception was calculated as % MPE (percent maximum possible effect) = (test latency-control latency/(10 s − control latency) × 100 for each dose tested. Cut-off time was 10 s.

In the naloxone antagonism test, naloxone was given 10 min before the (+)- or (−)-thebaine ED_{80} (dose producing 80% increase in MPE) was injected and latencies were measured 20 min later. For each point of the dose–response curve, percent antagonism was calculated as [1-(Naloxone + (+)- or (−)-thebaine MPE)/((+)- or (−)-thebaine MPE ED_{80})] × 100).

For the opioid antagonist subtype tests, β-funaltrexamine was given i.c.v. 4 h before either thebaine enantiomer. Nor-binaltorphimine and naltrindole pretreatment times were 2 h and 20 min, respectively and the data were treated as indicated above for the naltrexone antagonist test.

2.1.3. Phenylquinone abdominal-stretching assay

The procedure described by Pearl and Harris (1966) with modifications as indicated below was used. Six mice were injected per dose of test drug or vehicle and 10 min later received 2 mg/kg intraperitoneally (i.p.) of a freshly prepared paraphenylquinone solution. They were then placed in three cages in groups of two each. Then, the total number of stretches observed per group during each 1-min period was counted at 10 and 15 min. The total number of stretches for the three groups was determined. A stretch was characterized by an elongation of the mouse's body, development of tension in the abdominal muscles, and extension of the hindlimbs. The antinociceptive response was expressed as percent inhibition of the paraphenylquinone-induced stretching response and was calculated as [1 − (total number of stretches in the medicated mice)/(total number of stretches in the control mice)] × 100.

2.1.4. Hot-plate test

The method originally described by Eddy and Leimbach (1953) was used. Modifications are indicated below. A modified 1000 ml Pyrex beaker (bottom removed) was placed on the hot plate maintained at 56°C. A mouse was placed in the specially designed beaker. This arrangement served to confine a mouse to a specific area of the hot plate. Each mouse was exposed to the hot plate for two trials spaced 5 min apart. Only mice that gave a control response latency in the range of 6 to 10 s on both trials served as subjects. Each subject received a dose of test drug and 30 min later was again tested on the hot plate. Activity was scored as positive if the mouse jumped, licked or shook its paws at least 5 s beyond its average control latency. Cut-off time was 15 s. Percent activity for each dose tested was calculated as (total number of mice scored as positive)/(total number tested) × 100.

2.2. In vitro binding studies

2.2.1. Cell culture

A clonal Chinese hamster ovary (CHO) cell line, rat δ-opioid receptor (rDOR4), expressing the δ-opioid receptor was made using standard techniques. A clonal CHO cell line, mouse μ-opioid receptor (mMOR), expressing the mouse μ-opioid receptor was kindly provided by Duane Keith, Daniel Kaufman, Tuyet Tran and Chris Evans at the University of California at Los Angeles, USA. Both cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) + 10% fetal calf serum + 0.3 mg/ml G418 (LTI, Gaithersberg, MD).

2.2.2. Receptor binding studies

[^{3}H]Diprenorphine was used to measure opioid receptors in P_2 membrane preparations (36 Ci/mmol, Amersham, Arlington Heights, IL). P_2 membranes were prepared using standard techniques. Membranes (5–15 μg protein) were added to tubes containing [^{3}H]diprenorphine in 25 mM HEPES, pH 7.7 and incubated for 90 min at 24°C. Solutions were titrated with KOH (and not NaOH). Binding was terminated by rapid filtration through Whatman GF/B filters in a cell harvester. Non-specific binding was determined with 1 μM diprenorphine. Competitive assays were performed using 1 nM [^{3}H]diprenorphine and six concentrations (0.1 nM–10 μM) of displacing ligands, in the absence or presence of 150 mM NaCl. Assays were

Table 1
Antinociceptive effects of the optical isomers of thebaine in the tail-flick, paraphenylquinone and hot-plate tests in the mouse

Assay	Results: ED_{50} or AD_{50} (95% C.L.) in mg/kg	
	(−)-Thebaine	(+)-Thebaine
(1) Tail-flick agonist test	Inactive at 1, 3, 10, and 20 5/6 mice died of convulsions at 20	8.9 (3.4–22.1)
(2) Tail-flick antagonist test.	Pretreatment with 0.1 or 3.0 of naloxone followed by 20 of (−)-thebaine resulted in convulsions and death in 5/6	0.06 (0.015–0.43)
(3) Phenylquinone agonist test	Inactive at 1, 3 and 10	1.9 (1.6–9.5)
(4) Hot-plate agonist test	Inactive at 1, 3 and 10	22.9 (10.9–48.1)

performed in triplicate, and results represent the combined data of two or more independent experiments.

2.3. Data analyses

The determination of ED_{50}s ED_{80}s or AD_{50}s by least linear squares was accomplished using the StatView 512 + statistical package (Brainpower, Agoura Hills, CA). The K_i values were determined using the GraphPad Prism program (GraphPad, San Diego, CA).

2.4. Pharmacological agents

(−)-Thebaine · HCl, nor-binaltorphimine · HCl, and naloxone · HCl were supplied by the National Institute on Drug Abuse. (+)-Thebaine was prepared by one of us (KCR) using modifications of methodology previously described (Rice, 1980 and references cited therein). Naltrindole · HCl, and β-funaltrexamine · HCl were obtained commercially from Research Biochemicals International, Natick, MA.

3. Results

3.1. Antinociception and opioid–receptor antagonist interactions

The results in the tail-flick, paraphenylquinone and hot-plate tests are summarized in Table 1. As noted previously (Aceto et al., 1977; Tortella et al., 1984), (−)-thebaine was inactive antinociceptively and produced convulsions which were lethal. Apparently, naloxone offered only limited protection, if any, from the convulsive and lethal effects implying lack of opioid involvement. In sharp contrast, the unnatural isomer was active on all three analgesic assays and free of convulsive effects at the doses tested. It was approximately 0.2 as active as morphine in the tail-flick and phenylquinone tests and, curiously, had only 0.03 the activity of morphine in the hot-plate test. Naloxone blocked antinociception in the tail-flick test suggesting opioid-like activity.

The results of the opioid receptor-subtype experiments are presented in Table 2. Since (−)-thebaine was inactive antinociceptively, interactions with receptor antagonists could not be studied. However, it should be noted that naltrindole did not block the convulsions and lethality associated this natural enantiomer. Regarding (+)-thebaine, the results indicate that the antinociception obtained with this enantiomer is associated with μ- and δ- but not κ-opioid–receptor systems.

3.2. Binding studies

These assays were conducted in two separate cell lines. The mouse μ-opioid receptor (mMOR) or the rat δ-opioid receptor (rDOR) was stably expressed in CHO cells. We found that the CHO cells lacked any opioid receptors prior to transfection, as has been previously shown (Abood et al., 1995). Although diprenorphine, the radioligand in these experiments, is a non-selective opioid receptor antagonist, the cells are expressing individual cloned receptors, which are by definition, a single subtype.

Scatchard analysis of saturation isotherms with the transected cell lines revealed saturable, high-affinity binding. The mMOR cell line bound diprenorphine with a B_{max} of 7.58 ± 0.8 pmol/mg protein and a K_d of 0.42 ± 0.04 nM (Abood et al., 1995). The rDOR line bound diprenorphine

Table 2
Interactions of (−)- or (+)-thebaine with opioid receptor antagonist subtypes in the tail-flick assay

Opioid receptor antagonist subtype	Results: AD_{50} (95% C.L.)	
	(−)-Thebaine	(+)-Thebaine
μ: β-Funaltrexamine (μg/brain)	Not applicable	2.9 (0.8–10.98)
κ: nor-Binaltorphimine (mg/kg)	Not applicable	0% at 1, 10 and 30
δ: Nalrindole (mg/kg)	Not applicable; naltrindole pretreatment at 1 and 10 followed by 20 of (−)-thebaine resulted in 5/6 convulsions and death	3.8 (1.9–7.8)

Table 3
Summary of results of displacement binding studies with (−)- and (+)-thebaine in cell lines expressing transfected μ- and δ-opioid receptors

Cell line	Thebaine	K_i (μM)	% Displacement
mMOR	(−)-isomer	–	37% at 10 μM
	(+)-isomer	2.75 ± 0.01	65% at 10 μM
rDOR	(−)-isomer	1.02 ± 0.01	45% at 10 μM
	(+)-isomer	–	28% at 10 μM

with a B_{max} of 1.16 ± 0.9 pmol/mg protein and a K_d of 0.51 ± 15 nM [ibid]. Diprenorphine binding to the cell lines was comparable to a single site (Hill coefficients of.98 ± 0.05 and 1.05 ± 0.05 for the mMOR and rDOR cell lines, respectively). As summarized in Table 3, the displacement experiments showed significant competition at micromolar concentrations for [^{3}H]diprenorphine binding with both thebaine enantiomers in both mMOR and rDOR cell lines. At the μ-opioid receptor, the (+)-isomer was more effective than the (−)-isomer; a K_i value of 2.75 ± 0.01 μM was calculated with 65% displacement of [^{3}H]diprenorphine at 10 μM. At the δ-opioid receptor, a K_i value of 1.02 μM was obtained for (−)-thebaine, with 45% displacement of [^{3}H]diprenorphine at 10 μM.

4. Discussion

The pharmacology of (−)-thebaine was reviewed by a WHO Advisory Group (1980); it was reported to be free of morphine-like agonist properties. Earlier, we reported that natural thebaine lacked antinociceptive properties in the mouse, produced lethal convulsions, and did not replace morphine in abruptly withdrawn, morphine-dependent rhesus monkeys (Aceto et al., 1977). In the study reported here, we confirmed the previous findings in the mouse and determined that the convulsions and lethality were not blocked by naltrindole thereby absolving the δ-opioid system. At best, naloxone was only partly effective in this regard suggesting possible indirect involvement of the opioid system. In fact, (−)-thebaine was shown to inhibit binding to the glycine receptor and, to a lessor extent, to the γ-aminobutyric acid receptor (Goldfinger et al., 1981). Additionally, both receptors were implicated in the convulsive effects of opiates (Dingledine et al., 1978).

In their report, the WHO Advisory Group (1980), speculated that some of (−)-thebaine's potential abuse potential was due, at least partially, to (−)-oripavine, a metabolite. Recently, we reported that (−)-oripavine showed significant antinociceptive activity in mice and that μ-and δ-opioid receptor mechanisms were implicated (Aceto et al., 1998). We noted that the accompanying convulsions and lethality at higher doses could not be μ- or δ-opioid receptor related. This observation also supports the presumption that δ-opioid receptor agonists are not necessarily associated with convulsant activity. Finally, we hasten to add that the rapid onset of convulsions after (−)-thebaine, precluded the involvement of its metabolite (−)-oripavine.

On the other hand, (+)-thebaine elicited an entirely different profile of activity. In vivo, it produced antinociception whose mode of action related to μ- and δ- but not κ-opioid receptor interactions. The results of binding studies are in general agreement with the in vivo data if one allows for the fact that binding studies do not necessarily predict potency or efficacy. That convulsions were not observed in mice with the (+)-isomer at doses to 30 mg/kg, again demonstrating that μ- and especially δ-opioid receptor-induced antinociception could be dissociated from convulsive effects.

Other examples of anomalous activity associated with (+)-isomers of opioids have been observed. Opioid activity expressed as antinociception in rodents and physical dependence liability in rhesus monkeys with the 6-ethyl or 6-propyl (+)-homologs of metazocine (using the benzazocine nomenclature) was reported (Ager et al., 1969). Using Drieding stereomodels, Aceto and Zenk (1986) noted that if the piperidine ring of (+)-6,11 dialkylmetazocine was superimposed on the piperidine ring of (−)-morphine, then the ethyl and propyl chains at carbon 6 of these homologs could rotate in the region of the C ring of morphine. A similar explanation also applied regarding the (+)-homologs of N-substituted phenylmorphans which were also reported to induce analgesia and morphine-like physical dependence (Awaya et al., 1984). Then, we demonstrated that the piperidine rings of (−)-morphine and (+)-morphine were superimposable. As a result carbon atoms 3, 4 and 13 of (+)-morphine would align themselves with carbon atoms 3, 4 and 13, respectively of natural morphine, and the oxygen bridges overlapped. Rings A and B of both enantiomers appeared hinged; the rings gradually diverged from one another so that the widest separation between rings B was 1.5 Å and the widest separation between rings A was 2.0 Å. Importantly, the crucial difference was that rings C projected in opposite directions. Since μ-opioid receptor-induced antinociception and physical dependence (expressed as absolute stereospecificity) were associated with the C ring of the (−)-isomer of morphine and not with the C ring of (+)-morphine, we proposed that morphine-like activity required the C ring orientation of natural morphine. Obviously, this proposal does not accommodate (+)-thebaine's activity. Since the C rings of (+)-morphine and (+)-thebaine are not alike, this suggests the possibility that other μ- and δ-opioid receptor sites associated with (+)-thebaine's C ring orientation exist.

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