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European Journal of Pharmacology 470 (2003) 27–31
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Spinally mediated analgesia and receptor binding affinity of epibatidine analogs

Tomoki Nishiyama[a,*], Laszlo Gyermek[b], Mark L. Trudell[c], Kazuo Hanaoka[a]

[a] Department of Anesthesiology, The University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo, 113-8655, Japan
[b] Department of Anesthesiology, Harbor-University of California Los Angeles Medical Center, 1000 West Carson Street, Torrance, CA, 90509, USA
[c] Department of Chemistry, University of New Orleans, 102 Chemical Sciences Building, 2000 Lakeshore Drive, New Orleans, LA, 70148, USA

Received 27 January 2003; received in revised form 23 April 2003; accepted 29 April 2003

Abstract

Two epibatidine derivatives, (1R, 2R, 5S)-A-(2-chloropyridinyl) azabicyclo [3.2.1] octane; A=2β: analog 1, 2α: analog 2, were investigated for their spinally mediated analgesic effects and binding affinity to nicotinic acetylcholine receptors. The tail flick response and behavioral side effects were studied after intrathecal agents in rats. The membrane preparations of the Torpedo Californica and rat cerebral cortices were used for radioligand binding utilizing [^{3}H] epibatidine displacement. Their affinity to muscular and neuronal nicotinic acetylcholine receptors and spinally mediated analgesic potencies were 15, 20, and 3.8 times (analog 1) and 2000, 30,000, and 3.3 times (analog 2) less than epibatidine, respectively. Two times the analgesic 50% effective doses (ED_{50}s) of the analogs did not induce side effects, while one-third of that of epibatidine induced motor disturbance. In summary, the two epibatidine analogs have higher potency ratio of spinally mediated analgesia/side effects than epibatidine.

Keywords: Nicotinic acetylcholine receptor; Epibatidine; Radioligand; Analgesia; Spinal cord

1. Introduction

A potent antinociceptive effect has been shown after intracerebroventricular (Molinero and Del Rio, 1987) or intrathecal (Aceto et al., 1986) administration of nicotine. Nicotinic cholinergic receptors in the spinal cord seem to have an important role in the modulation of persistent pain (Lawand et al., 1999).

Compared to (−)-nicotine, (±)-epibatidine is approximately 200-fold more potent in producing analgesia in the mice hot plate test (Sullivan et al., 1994a). The antinociceptive effect of both nicotine and epibatidine is of rapid onset (3–5 min), but whereas the action of nicotine is short-lived (10 min), the effect of epibatidine may last up to 1 h (Bannon et al., 1995; Qian et al., 1993). However, 65% mortality was observed in 5 days in mice that received repeated (±)-epibatidine treatment (Sullivan et al., 1994a). Epibatidine is extremely toxic causing respiratory paralysis, seizures, and death after doses only slightly higher than those required for antinociception (Bonhaus et al., 1995; Sullivan et al., 1994b). Separation of the analgesic properties from such side effects would represent useful innovation.

Two epibatidine derivatives, (1R, 2R, 5S)-2β-(2-chloropyridinyl) azabicyclo [3.2.1] octane and (1R, 2R, 5S)-2α-(2-chloropyridinyl) azabicyclo [3.2.1] octane, were synthesized, which were less potent in nicotinic receptor binding and nicotinic stimulant effects on arterial pressure (Zhang et al., 1997) and on isolated intestinal segments (Ji et al., 2000, personal communication) than epibatidine. Should these epibatidine analogs be relatively more potent in analgesic effects than epibatidine, they may become useful leads for obtaining new analgesics with better therapeutic index. In the present study, we re-evaluated the binding affinity of these agents to the nicotinic receptors and examined their analgesic potencies in comparison with epibatidine.

2. Materials and methods

The protocol was approved by the research committee of The University of Tokyo, Harbor-University of California

* Corresponding author. 3-2-6-603, Kawaguchi, Kawaguchi-shi, Saitama, 332-0015, Japan. Tel.: +81-3-5800-8668; fax: +81-3-5800-9655.
E-mail address: nishit-tky@umin.ac.jp (T. Nishiyama).

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doi:10.1016/S0014-2999(03)01784-9

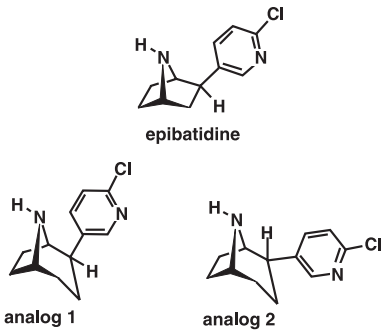

Fig. 1. Structures of epibatidine, analogs 1 and 2.

Los Angeles Medical Center, and University of New Orleans.

2.1. Agents

Epibatidine (Sigma, St. Louis, MO), [^{3}H] epibatidine (Amersham Pharmacia, Piscataway, NY), mecamylamine (Sigma), (1R, 2R, 5S)-2β-(2-chloropyridinyl) azabicyclo [3.2.1] octane (analog 1), and (1R, 2R, 5S)-2α-(2-chloropyridinyl) azabicyclo [3.2.1] octane (analog 2) were used in this study. These two agents were derived from natural (−)-cocaine (Zhang et al., 1997). Their structures are shown in Fig. 1.

2.2. In vitro ligand binding tests

For determining binding affinity to the muscular type nicotinic receptor, Torpedo Californica (Pacific Biomarine, Venice, CA) membranes were prepared from the electric organ by homogenization and three step centrifugation (e.g. first low speed 4000 × *g* and subsequently two high speed 40,000 × *g* phases) at 0–4 °C. Aliquots of the membrane preparation (representing 0.1–0.2 mg protein content) were used for radioligand binding utilizing [^{3}H] epibatidine displacement in a conventional ligand binding setup (Kanne and Abood, 1988). Displacing ligands (analog 1:0.003, 0.03, 0.3, 1, 3, and 30 μg; analog 2:0.1, 0.3, 1, 10, 30, and 100 μg), using four samples at each concentration were incubated with [^{3}H] epibatidine for 90 min at room temperature. The binding was terminated by centrifugation (12,000 × *g* for 10 min). The cutoff tips of the microtubes containing the ligands were placed in scintillation vials to which tissue solubilizer (TS-2, Research Products International Mount Prospect, IL) and scintillation fluid (Ready Flow III, Beckman Instruments Fullerton, CA) were added. Beta emission was counted within 24 h with 40% efficiency by scintillation counting (Scintillation Counter Packard Tricarb 2200, Packard Instrument, Meridien, CT). Fifty percent inhibitory concentration (IC_{50}) was calculated from the nonlinear regression curves automatically with the Prism 2™ (Graph Pad, San Diego, CA).

For determining binding affinity to neuronal nicotinic receptors, ligand-binding tests were performed using rat cerebral (Harlane Laboratories, Indianapolis, IN) cortical membranes. The binding test was otherwise similar to that described for the Torpedo tissue membrane.

2.3. In vivo intrathecal analgesic test

Male Sprague–Dawley rats of approximately 350 g weight (Nippon Bio Supply, Tokyo, Japan) were implanted with lumbar intrathecal catheters (PE-10; Clay Adams, Parsippany, NJ) under halothane (2%) anesthesia (Yaksh and Rud, 1976). Behaviorally normal rats were studied 7 days later.

The tail flick test and behavioral study were performed after intrathecal injection of the agent. Six rats were used in each dose group. Epibatidine (3, 10, 30, and 100 ng), and analogs 1 and 2 (40, 100, and 250 ng) dissolved in normal saline were administered in 10 μl. Saline 10 μl, was administered as control. The effects of intraperitoneal administration of mecamylamine (0.7 mg) followed immediately by intrathecal administration of epibatidine (30 ng), analog 1 or analog 2 (100 ng) was also tested.

For the tail flick test, noxious stimulation was provided by a beam of high intensity light (Tail-flick Analgesia Meter MK-330A, Muromachi Kikai, Tokyo, Japan) focused on the tail (Nishiyama et al., 2001). The response time was measured and defined as the interval between the onset of the thermal stimulation and the abrupt flick of the tail. The cutoff time in the absence of a response was set to 14 s to prevent tissue injury. Data were processed as the % maximum possible effect (%MPE): [(postdrug latency − baseline latency)/(cutoff time (14 s) − base line latency)] × 100. The 50% effective dose (ED_{50}) was calculated with a computer program made by Takano (Department of Anesthesiology, University of California San Diego, CA) based on the book by Tallarida and Murray (1981) as the dose

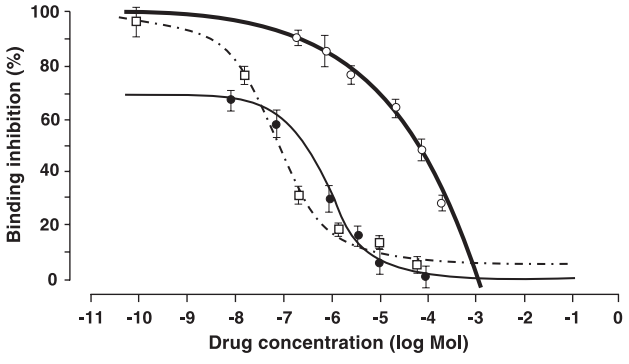

Fig. 2. The dose–response inhibition of the receptor binding with Torpedo membranes. Closed circle, analog 1; open circle, analog 2; open square, epibatidine. Bars indicate S.E. Each four samples of epibatidine 0.00003, 0.003, 0.1, 0.3, 3, and 30 μg; analog 1 0.003, 0.03, 0.3, 1, 3, and 30 μg, and analog 2 0.1, 0.3, 1, 10, 30 and 100 μg were used. [^{3}H] epibatidine was used in the range of 1–10 nC.

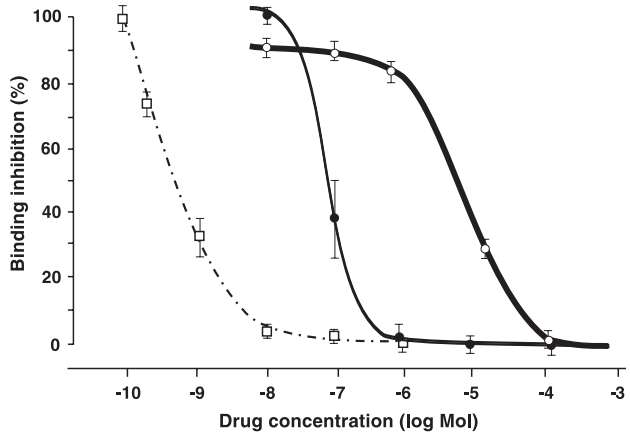

Fig. 3. The dose response inhibition of the receptor binding with rat cerebral cortical membranes. Closed circle, analog 1; open circle, analog 2; open square, epibatidine. Bars indicate S.E. Each four samples of epibatidine 0.00003, 0.0001, 0.0003, 0.003, 0.03, and 0.3 μg; analog 1 0.003, 0.03, 0.3, 1, 3, and 30 μg; and analog 2 0.003, 0.03, 0.3, 1, 3, and 30 μg were used. [^{3}H] epibatidine was used in the range of 0.3–100 nC.

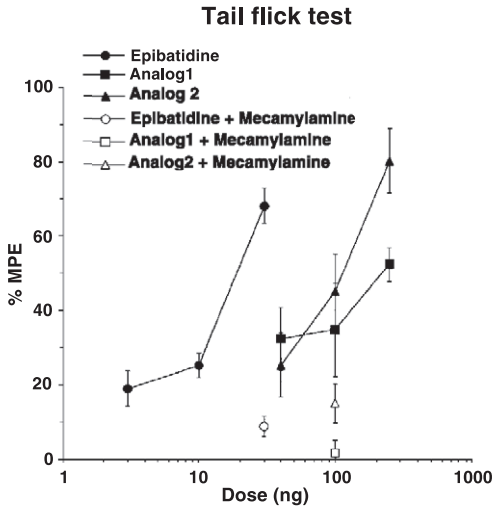

Fig. 4. Analgesic effects of the agents with or without mecamylamine in the tail flick test. Bars indicate S.E. %MPE: % maximum possible effect calculated as [(postdrug latency – baseline latency)/(cutoff time (14 s) – baseline latency)] × 100. Six rats were used in each dose group. Epibatidine (3, 10, 30, and 100 ng), and analogs 1 and 2 (40, 100, and 250 ng) were administered in 10 μl. Saline 10 μl was administered as control. The effects of intraperitoneal administration of mecamylamine (0.7 mg) followed immediately by intrathecal administration of epibatidine (30 ng), analog 1 or analog 2 (100 ng) was also tested.

that produces a value of 50% MPE using each maximum effect.

The general behavior (including agitation and allodynia), motor function, flaccidity, pinna reflex, and corneal reflex were observed by a blinded investigator. They were judged as present or absent. Further details of the test were described previously (Nishiyama et al., 2001). Briefly, agitation was judged as spontaneous irritable movement and/or vocalization. Allodynia was judged as an evoked agitation by lightly stroking the flank of the rat. Motor function was evaluated by: (a) placing/stepping and (b) righting reflexes. The former was evoked by drawing the dorsum of either hind paw across the edge of the table. The latter was assessed by placing the rat horizontally with its back on the table. Flaccidity was judged as a result of muscle weakness. Pinna and corneal reflexes were elicited by a narrow paper string.

3. Results

3.1. In vitro ligand binding tests

The dose-related response inhibition of the nicotinic receptor binding to Torpedo membranes is shown in Fig. 2 and to rat cerebral cortical membranes in Fig. 3. The IC_{50} values are shown in Table 1. The affinities of analogs 1 and 2 to muscular nicotinic acetylcholine receptors were 15 and 2000 times, and those to neuronal nicotinic acetylcholine receptors were 20 and 30,000 times lower than that of epibatidine, respectively.

3.2. In vivo intrathecal analgesic test

Epibatidine and both analogs exhibited dose-dependent analgesic effects in the tail flick test (Fig. 4). Analogs 1 and 2 had similar ED_{50}s, which were 3.8 and 3.3 times larger than that of epibatidine. respectively (Table 2). Treatment with mecamylamine decreased the tail flick latency compared to epibatidine, analog 1 or analog 2 alone.

Agitation and allodynia were seen in the rats receiving 30 and 100 ng of intrathecal epibatidine (Fig. 5). Epibatidine 10 and 100 ng induced motor disturbance. Flaccidity and loss of pinna or corneal reflexes were not observed in

Table 1
Binding study

Agent	Torpedo membranes	Rat cortex membranes
Epibatidine	7.12 ± 0.07	9.55 ± 0.28
Analog 1	5.86 ± 0.10	8.27 ± 0.43
Analog 2	3.80 ± 0.17	5.06 ± 0.12

Values are indicated as—log (Mol). IC_{50} values (± S.E.) of [^{3}H] epibatidine displacement are shown. N = four samples in each concentration of the agent.

Table 2
Intrathecal analgesic test

Agent	ED_{50} (ng ± S.E.)
Epibatidine	32.0 ± 2.2
Analog 1	120.7 ± 8.7
Analog 2	105.1 ± 5.2

Fifty percent effective doses (ED_{50}s) are shown.
N = six rats in each concentration of the agent.

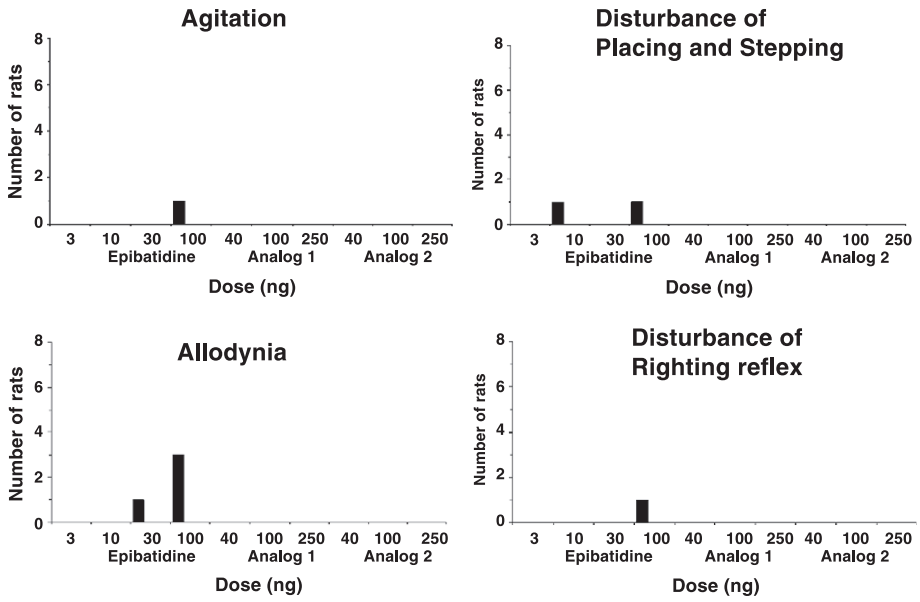

Fig. 5. Side effects. Number of rats with each side effect is shown. Total number of rats tested in each dose was six. Only the epibatidine induced side effects, but these were reversible.

this study. Analogs 1 and 2 in doses up to 250 ng did not induce any behavioral and motor disturbances.

4. Discussion

Analog 1 shows 15 and 20 times and analog 2 shows 2000 and 30,000 times less affinity to muscular and neuronal nicotinic acetylcholine receptors, respectively. However, spinally mediated analgesic potency to acute thermal stimulation of analogs 1 and 2 was only 3.8 and 3.3 times less than that of epibatidine, respectively. In addition, two times doses of the ED_{50} of analogs 1 and 2 did not induce observable side effects, while already one-third dose of the ED_{50} of epibatidine induced motor disturbance.

The dose–response inhibition curves of the analogs 1 and 2 in Fig. 1 and the curve of the analog 2 in Fig. 2 did not begin/end at 0%/100%. However, if more data points with lower and/or higher doses were added, the curves might begin and/or end at 0% and/or 100%.

The full expression of nicotine-induced antinociception in the rat appears to involve descending monoaminergic pathways to the lumbar spinal cord, as well as intrinsic spinal muscarinic interneurons (Rogers and Iwamoto, 1993). The potential therapeutic value of nicotinic acetylcholine receptor agonists for the treatment of persistent or chronic pain may depend on obtaining receptor subtype selectivity for analgesia vs. toxicity.

Epibatidine, an azabicycloheptane alkaloid, is a highly potent but nonselective stimulant of neuronal nicotinic acetylcholine receptors (Sullivan and Bannon, 1996). Spinal administration of epibatidine inhibits the development of hyperalgesia and inflammation (Lawand et al., 1999). Systemically administered epibatidine was antinociceptive in foot shock and hot plate stimulation, which was reversed by the nicotinic receptor antagonist mecamylamine but not by the opioid receptor antagonist naloxone (Bonhaus et al., 1995). The action of epibatidine is non-opioid but nicotinergic. Epibatidine's analgesic effect can be attenuated by pre-treatment with the centrally acting nicotinic acetylcholine receptor antagonist mecamylamine, but not with the peripherally acting nicotinic acetylcholine receptor antagonist hexamethonium (Sullivan et al., 1994a). This suggests that the analgesic effect of epibatidine is mediated by at least one type of central nicotinic acetylcholine receptors. The racemic 8-azabicyclo[3.2.1]octane homoepibatidine derivative exhibits efficacy almost equal to that of epibatidine, and its antinociceptive responses were blocked by pretreatment with mecamylamine (Xu et al., 1996). Another epibatidine derivative, (*R*)-5-(2-azetidinylmethoxy)-2-chloropyridine, is less potent than (+)-epibatidine in assays of acute and persistent pain and in the rotarod assay. Epibatidine and its analogs showed analgesic effects in the present study by intrathecal administration, which suggests they act on nicotinic acetylcholine receptors in the spinal cord. In the present receptor binding study, we did not use the spinal cord. Therefore, we cannot discuss the analgesic potency in relation with the affinity to the receptor.

Regarding toxic effects, it is known that convulsions, hypertension, and hypothermia produced by neuronal nicotinic acetylcholine agonists or (−)-nicotine maybe mediated via actions at the neuronal, but not at the neuromuscular, nicotinic acetylcholine receptors (Boyce et al., 2000). Systemically administered 10 μg/kg epibatidine produced apparent sedation and 50 μg/kg induced convulsions and death in mice (Bonhaus et al., 1995). Further-

more, intravenously administered epibatidine produced a transient increase in blood pressure and splanchnic sympathetic nerve discharge (Fisher et al., 1994). Intrathecal (−)-epibatidine also elicited dose-dependent increases in pressor- and heart-rate responses in rats, as well as an increase in the latency to withdraw from noxious thermal stimuli (Khan et al., 1997). An epibatidine derivative, (*R*)-5-(2-azetidinylmethoxy)-2-chloropyridine, less potent than (+)-epibatidine displayed a clearer separation between its motor and anti-hyperalgesic effects than (+)-epibatidine (Kesingland et al., 2000). The azabicyclo[2.2.1] heptene analog of epibatidine binds to rat native α4β2 with affinity comparable to that of nicotine and possesses similar potency as nicotine, but exhibits lower efficacy, in stimulating dopamine release from rat striatum (Bencherif et al., 1996). The maximal effects of this analog on neuronal ganglionic and muscular nicotinic acetylcholine receptor subtypes are also substantially smaller than those of nicotine (Bencherif et al., 1996). In the present study, we did not measure blood pressure or sympathetic activity. However, no sedation, convulsion or death was seen with the doses used in the present study. Of the two analogs, analog 2 had significantly lower affinity to muscular and neuronal nicotinic acetylcholine receptors and lower incidence of side effects than (±)-epibatidine. In contrast, only slight differences were observed in spinally mediated analgesic potency to acute thermal stimulation. Since lesser side effects related to peripheral and central nicotinic acetylcholine receptors would be expected with the analogs than epibatidine. These would have a wider therapeutic window than epibatidine.

In conclusion, the ratio between spinally mediated analgesic potency and side effects might be significantly with smaller in the presently described epibatidine analogs than in epibatidine.

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