0026-895X/94/040563-07$03.00/0

MOLECULAR PHARMACOLOGY, 45:563-569

ACCELERATED COMMUNICATION

Epibatidine, a Potent Analgetic and Nicotinic Agonist

BARBARA BADIO and JOHN W. DALY

Laboratory of Bioorganic Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892

Received December 1, 1993; Accepted January 13, 1994

SUMMARY

Synthetic (+)- and (−)-epibatidine (an alkaloid originally characterized from frog skin) have potent analgetic activity in mice, using the hot-plate assay. The natural (+)-enantiomer, with an ED_{50} of about 1.5 μg/kg upon intraperitoneal injection, is about 2-fold more potent than the (−)-enantiomer. The analgetic activity is blocked by the nicotinic antagonist mecamylamine. Both the (+)- and (−)-enantiomers have high affinity (K_i values of 0.045 and 0.058 nM, respectively) for nicotinic sites that bind [^{3}H] nicotine in rat brain membranes. An analog of epibatidine with the chloro substituent of the pyridyl ring replaced with hydrogen has comparable affinity for nicotinic sites, whereas replacement with a methyl or iodo substituent lowers activity. Both (+)- and (−)-epibatidine have potent agonist activity at ganglionic-type nicotinic receptors in pheochromocytoma PC-12 cells, with EC_{50} values for stimulation of sodium influx of 72 and 111 nM, respectively. (−)-Epibatidine is about 5-fold less potent as an agonist at muscle-type central nicotinic receptors of medulloblastoma TE671 cells. It would appear that the analgetic activity of epibatidine is due to activity as a nicotinic agonist. The epibatidines have little or no activity at a variety of other central receptors, including opioid receptors, muscarinic receptors, adrenergic receptors, dopamine receptors, serotonin receptors, and γ-aminobutyric acid receptors.

The structure of a novel azabicycloheptane alkaloid was recently elucidated and the compound was named epibatidine, in reference to the Ecuadorean frog *Epipedobates tricolor* from which it was isolated in trace amounts (1). Epibatidine was a potent analgetic agent whose *in vivo* activity was not antagonized by naloxone (1). Additional studies were not possible because of the lack of a significant source of the natural alkaloid. Synthesis of epibatidine has now been achieved by five laboratories (2–6). Synthesis of both racemic epibatidine and (+)- and (−)-epibatidine was reported. We have now initiated studies on the central sites of action of synthetic (+)- and (−)-epibatidine. Both enantiomers had potent analgetic activity, and the only central receptors at which potent activity was found were the nicotinic receptors. Structures of epibatidine and analogs are shown in Fig. 1.

Materials and Methods

Drugs. The enantiomers of epibatidine, i.e., (+)-**1a** and (−)-**1b**, and the three analogs **2**, **3**, and **4** were provided by Drs. Teck-Peng Loh and E. J. Corey (Harvard University, Cambridge, MA). The radioligands [^{3}H]nicotine (75.7 Ci/mmol), [^{3}H]DAMGO (52.5 Ci/mmol), [^{3}H] U69,593 (50.6 Ci/mmol), and [^{3}H]DPDPE (33.0 Ci/mmol) were from New England Nuclear (Boston, MA). β-Dihydroerythroidine, mecamylamine, and (−)-nicotine ditartrate were from Research Biochemicals, Inc. (Natick, MA), and *d*-tubocurarine was from Boehringer Mannheim (Mannheim, Germany).

Animals. Adult male NIH Swiss strain mice, weighing 25–30 g, were used. All drugs were dissolved in a 20:80 (v/v) mixture of Emulphor EL-620 (Rhône Poulenc, Cranbury, NJ) and 0.9% saline solution and were administered intraperitoneally in a volume corresponding to 5 ml/kg of body weight.

Hot-plate analgetic assay. The mice were placed on a metal plate that was heated to 55–56° and enclosed in a glass cylinder. Time to appearance of the first sign of pain (licking or shaking the hind paw, jumping, or climbing the sides of the cylinder) was measured. The procedure was based on that described by Eddy and Leimbach (7). The reaction time for each mouse without drug was determined twice by using a stopwatch. Each mouse was then given an intraperitoneal injection of test agent or agents. The reaction time of each mouse was determined at preset time intervals of 5, 10, 20, 30, 45, 60, 90, and 120 min.

Membrane preparation. Brains from rats or guinea pigs obtained from Pel Freez Biologicals (Rogers, AR) were placed in ice-cold 50 mM Tris·HCl buffer, pH 7.4. Tissue was homogenized using a Polytron homogenizer (setting 6, 10 sec). The homogenate was centrifuged for 15 min at 35,000 × *g* at 4°. The pellet was washed once by recentrifu-

ABBREVIATIONS: DAMGO, [D-Ala^2, *N*-methyl Phe^4, Gly-ol^5]enkephalin; DPDPE, [D-penicillamine^2, D-penicillamine^5]enkephalin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

gation in Tris buffer. The final pellet was resuspended in Tris buffer and stored at −70° until needed. Aliquots were suspended in the appropriate incubation buffer and used for receptor binding assays.

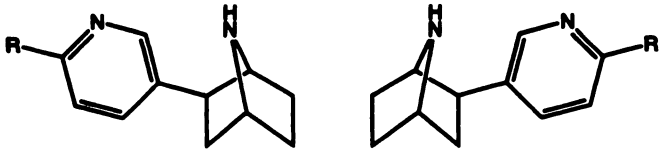
Binding assays. Membranes were diluted to a concentration of 1–3 mg/ml for binding assays. Protein concentrations were determined by the bicinchoninic acid protein assay (Pierce Chemical Co., Rockford, IL), using bovine albumin as a standard.

[^{3}H]Nicotine receptor binding was assayed with rat cerebral cortical membrane preparations by a modification of described procedures (8–10), in 20 mM HEPES buffer, pH 7.4, containing 1 mM $MgCl_2$, 120 mM NaCl, 5 mM KCl, and 2 mM $CaCl_2$. Each assay contained the test agent or agents added in 5 μl, a suspension of the rat cerebral cortical membranes (100 μl containing 200–300 μg of protein), 200 μM diisopropyl fluorophosphate, and 2 nM [^{3}H]nicotine, in a final volume of 0.5 ml. Each assay was for 120 min at 0–4° and was performed in triplicate. Nonspecific binding was determined with 10 μM nicotine. Binding reactions were terminated by filtration through Whatman GF/B filters, using a Brandel M24R cell harvester (Brandel, Gaithersburg, MD). Filters were washed twice with 5 ml of ice-cold buffer and placed in scintillation vials with 5 ml of Hydrofluor scintillation fluid, followed by counting for tritium. The filters were presoaked in 0.3% polyethylenimine to reduce nonspecific binding.

Opioid receptor binding was assayed in membrane preparations from rat brain (μ and δ receptors) or guinea pig cerebellum (κ receptors), at pH 7.4 in 50 mM Tris·HCl buffer, by a modification of described procedures (11). Each assay contained the agent or agents added in 5 μl, a 2 nM concentration of tritiated ligand, and a suspension of brain membranes (100 μl containing 400–500 μg of protein), in a final volume of 0.5 ml. Tritiated ligands were as follows: μ receptors, [^{3}H]DAMGO; δ receptors, [^{3}H]DPDPE; κ receptors, [^{3}H]U69,593. Each assay (60 min for μ and κ receptors, 120 min for δ receptors) was at 25° and was performed in triplicate. Nonspecific binding was determined with 10 μM naltrexone. Filtration, washing, and scintillation counting were as described above for the [^{3}H]nicotine binding assay. Filters were presoaked in 0.3% polyethylenimine to reduce nonspecific binding.

Serotonin receptor (5-hydroxytryptamine type 1 receptor) binding was assayed in rat cerebral cortical membrane preparations by a modification of described procedures (12), in 50 mM Tris·HCl buffer, pH 7.4, containing 4 mM $CaCl_2$ and 10 μM pargyline. Each assay contained 5 μl of the test agent or agents, a suspension of rat cerebral cortical membranes (100 μl containing 200–300 μg of protein), and 2 nM 5-[^{3}H]hydroxytryptamine, in a final volume of 0.5 ml. Each assay was for 10 min at 37° and was performed in triplicate. Nonspecific binding was defined in the presence of 10 μM 5-hydroxytryptamine. Filtration, washing, and scintillation counting were as described above for the [^{3}H]nicotine binding assay.

Muscarinic receptor binding was assayed in rat cerebral cortical membrane preparations by a modification of described procedures (13),



(+)-1a
R = Cl

(−)-1b
R = Cl

2: R = H
3: R = CH_3
4: R = I

Fig. 1. Structures of (+)- and (−)-epibatidine (**1a** and **1b**) and analogs (**2–4**). Natural epibatidine was found to be the enantiomer **1b** (negative rotation as free base, positive rotation as oxalate salt) by comparison of a sample of *N*-acetylepibatidine with *N*-acetyl derivatives of the two synthetic enantiomers (Dr. R. Baker, Merck Sharp and Dohme Research Laboratories, personal communication).

in 20 mM HEPES buffer, pH 7.4, containing 100 mM NaCl and 10 mM $MgCl_2$. Each assay contained 5 μl of the test agent or agents, a suspension of rat cerebral cortical membranes (100 μl containing 200–300 μg of protein), and 2 nM [^{3}H]quinuclidinyl benzilate, in a final volume of 0.5 ml. Each assay was for 30 min at 37° and was performed in triplicate. Nonspecific binding was defined in the presence of 1 μM atropine. Filtration, washing, and scintillation counting were as described above for the [^{3}H]nicotine binding assay.

Cultured cells. Pheochromocytoma PC-12 cells were provided by Dr. G. Guroff (National Institutes of Health, Bethesda, MD), and medulloblastoma TE671 cells were from the American Type Culture Collection (Rockville, MD). Pheochromocytoma PC-12 cells were grown in Dulbecco's modified Eagle medium with 6% fetal calf serum, 6% horse serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Medulloblastoma TE671 cells were grown in Dulbecco's modified Eagle medium with 10% fetal calf serum, 100 units/ml pencillin, and 100 μg/ml streptomycin. Cells were grown at 37° in an atmosphere enriched in CO_2.

Ion flux assays. Stimulation of sodium influx was assayed in cultured cells based on a described procedure (14). Cells were plated in six-well culture plates (coated with poly-D-lysine for PC-12 cells) and cultured with [^{3}H]leucine-containing medium for 24 hr (TE671 cells) or 48 hr (PC-12 cells). Medium was removed by aspiration and 0.5 ml of preincubation buffer (150 mM NaCl, 5.4 mM KCl, $CaCl_2$, 50 mM HEPES/Tris, pH 7.4, 5 mM glucose) was added at 22°. After 10 min, the preincubation buffer was replaced with influx buffer (50 mM NaCl, 5.4 mM KCl, $CaCl_2$, 50 mM HEPES/Tris, pH 7.4, 5 mM glucose, 179 mM sucrose, 5 mM ouabain) containing ^{22}NaCl and epibatidine or other agonists. Antagonists were present in both preincubation and influx buffers. After 2 min at 22°, the influx buffer was removed by aspiration and the cells were washed three times with wash buffer (same composition as preincubation buffer). Cells were solubilized with 0.5 ml of 1% sodium dodecyl sulfate in 0.5 N NaOH for 30–60 min and were pipetted into counting vials with 5 ml of Hydrofluor and 0.25 ml of 1 N HCl; radioactivity (^{3}H or ^{22}Na) was determined in a scintillation counter.

Data analysis. Dose-response curves were analyzed for EC_{50} and IC_{50} values by computer, using a nonlinear regression formula.

Results

(+)-Epibatidine caused significant hot-plate analgesia in mice at a dose of 2.5 μg/kg (Fig. 2A). (−)-Epibatidine was somewhat less potent, but a 5 μg/kg dose caused marked analgesia (Fig. 2B) comparable to that elicited by 10 mg/kg morphine (see Fig. 3B). The analgesia elicited by (−)-epibatidine was abolished by the nicotinic antagonist mecamylamine but was only slightly reduced by the nicotinic antagonist β-dihydroerythroidine (Fig. 3A). Mecamylamine produced no significant reduction in morphine-induced analgesia (Fig. 3B).

Both (+)- and (−)-epibatidine inhibited binding of [^{3}H]nicotine to rat cerebral cortical membranes (Fig. 4). The (+)-enantiomer was slightly more potent than the (−)-enantiomer. The K_i values were, respectively, 0.045 ± 0.004 and 0.058 ± 0.007 nM. An analog with the pyridyl chloro substituent replaced with hydrogen had a K_i value of 0.031 ± 0.002 nM, whereas analogs with methyl or iodo substituents were much less potent than epibatidine (Table 1).

Epibatidine at 10 μM had no effect on the binding of radioligands to μ-, δ-, or κ-opioid receptors, muscarinic receptors, or serotonin 5-hydroxytryptamine type 1 receptors (data not shown). Epibatidine also was assayed in the NOVA Screen (Hanover, MD) at 10 μM. No significant activity was found at adenosine, adrenergic, dopamine, γ-aminobutyric acid, serotonin, cholecystokinin, substance P, neurotensin, or excitatory

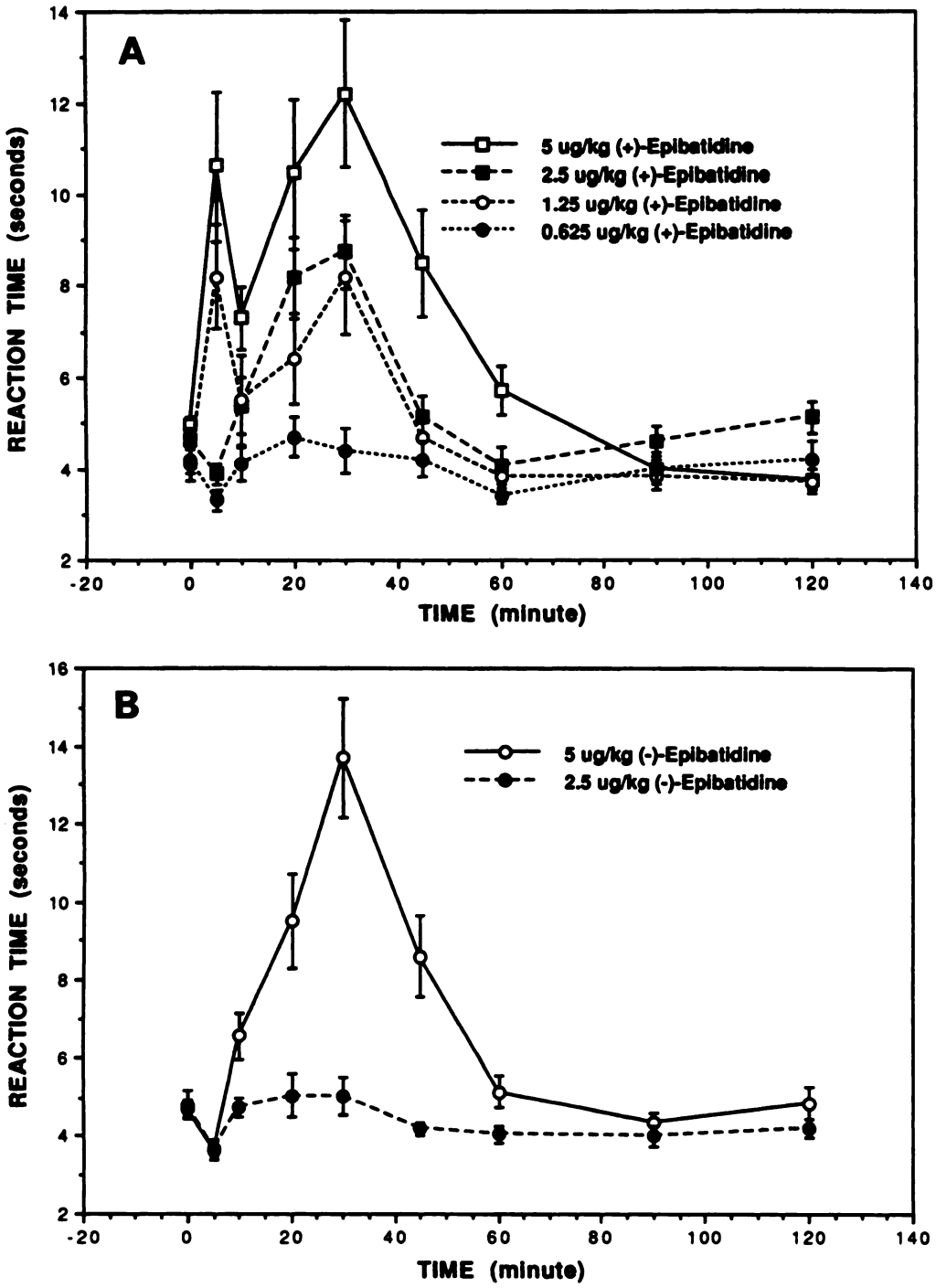


Fig. 2. Analgetic activity of (+)-epibatidine (A) and (−)-epibatidine (B) in mice. Hot-plate analgetic assays were as described in Materials and Methods. Each value is mean ± standard error (n = 10–20 animals).

amino acid receptors, at regulatory sites at γ-aminobutyric acid (benzodiazepine) or *N*-methyl-D-aspartate (MK-801) receptors, or at phencyclidine or σ sites (data not shown).

In rat pheochromocytoma PC-12 cells (+)- and (−)-epibatidine caused a marked stimulation of $^{22}Na^{+}$ influx (Fig. 5A). The EC_{50} values were 72 ± 7 and 111 ± 17 nM. (−)-Nicotine had an EC_{50} of 20,200 ± 1,800 nM. In rat medulloblastoma TE671 cells (−)-epibatidine had an EC_{50} of 528 ± 23 nM (Fig. 5B). (+)-Epibatidine was not tested because of limited supplies. Data with epibatidine oxalates in TE671 cells indicated that (+)-epibatidine is about 2-fold less potent than (−)-epibatidine. (−)-Nicotine had an EC_{50} of 60,300 ± 3,200 nM. The responses to (−)-epibatidine were blocked in both cell lines by nicotinic antagonists. For PC-12 cells the order of potency was mecamylamine = *d*-tubocurarine (Fig. 6A), whereas for TE671 cells the order of potency was *d*-tubocurarine ≫ mecamylamine (Fig. 6B). The IC_{50} values are given in Table 2.

Discussion

Elucidation of the structure of epibatidine, an alkaloid that has analgetic activity many-fold greater than that of morphine and that acts through naloxone-insensitive pathways (1),

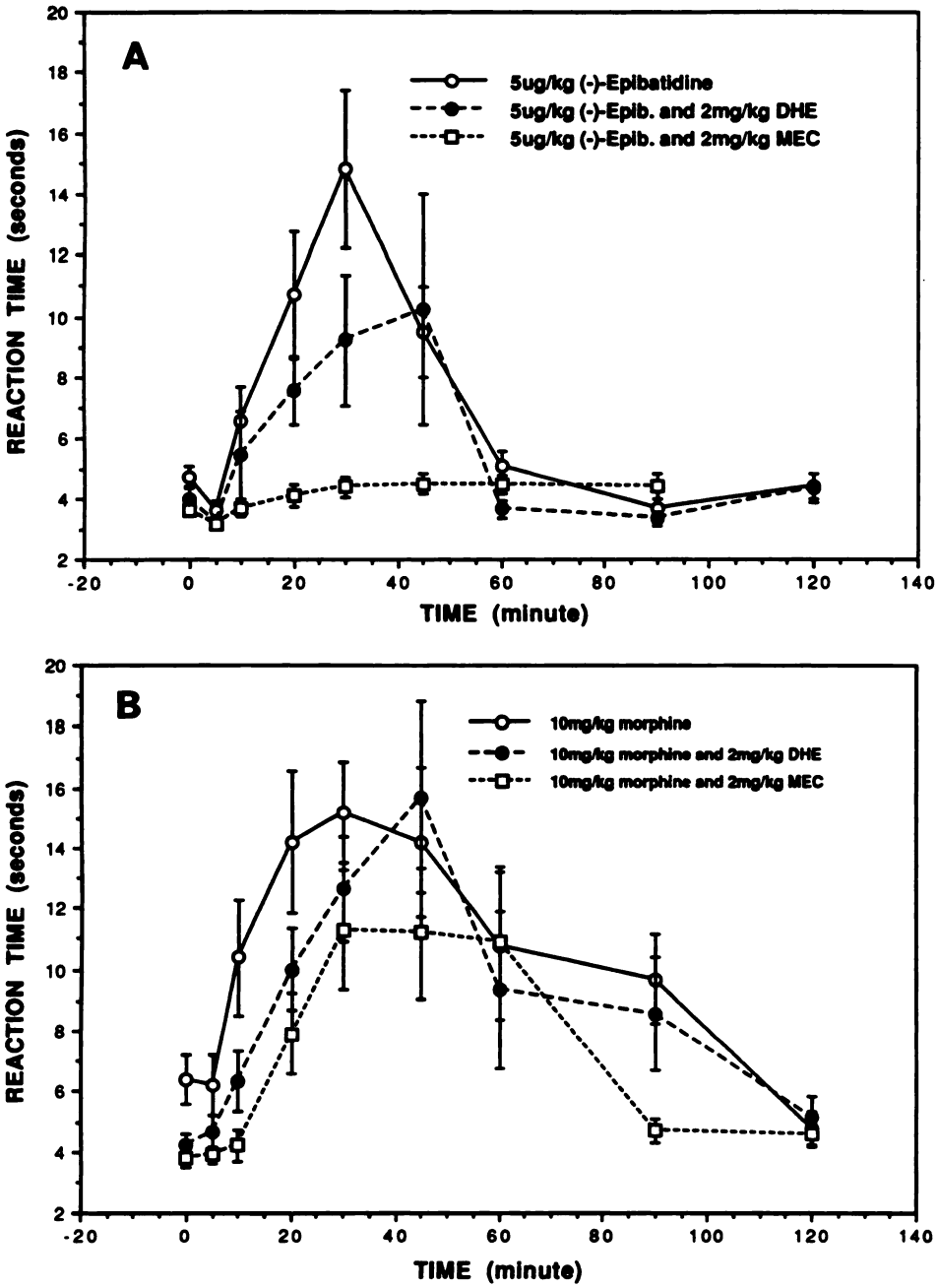


Fig. 3. Effect of nicotinic antagonists on (−)-epibatidine-elicited analgesia (A) and morphine-elicited analgesia (B). (−)-Epibatidine at 5 μg/kg or morphine at 10 mg/kg was adminstered 5 min after 2 mg/kg β-dihydroerythroidine (*DHE*) or 2 mg/kg mecamylamine (*MEC*). Hot-plate analgetic assays were as described in Materials and Methods. Each value is mean ± standard error (n = 10–20 animals).

prompted synthesis in several laboratories (2–6). The site of action of epibatidine has now been investigated. Epibatidine was found to act selectively as a potent agonist at nicotinic receptors. Such activity is perhaps not unexpected in view of structural similarities to nicotine. The epibatidines are about 20-fold more potent at central nicotine binding sites than is nicotine (Table 1). In contrast to the epibatidines, which show little enantiomeric selectivity (Table 1), nicotine exhibits marked enantiomeric selectivity for central nicotinic receptors, with the natural (+)-enantiomer being about 20-fold less active than the (−)-enantiomer (10). Thus, unlike that of nicotine, the mode of binding of epibatidine to nicotinic receptors must occur so that the relative position of the chloropyridyl ring is not crucial for activity. This is also true for the nornicotines, where the (+)- and (−)-enantiomers have equivalent affinities at [^{3}H]nicotine binding sites (10). The K_i values reported for (−)-nicotine were 1.4–1.9 nM, whereas (+)-nicotine and the two nornicotines all had K_i values in the 30–50 nM range (10). When (+)- and (−)-*N*-methylepibatidine are available, their activities will be of considerable interest. Epibatidine had no activity at a variety of other receptors (see Results).

The epibatidines are full agonists at nicotinic receptors, as demonstrated by effects on sodium influx in cultured cells expressing different subtypes of nicotinic receptors (Fig. 5). In pheochromocytoma PC-12 cells, containing a ganglionic-type nicotinic receptor (14), (+)- and (−)-epibatidine had comparable potencies, with EC_{50} values of 72 nM and 111 nM, respectively. The epibatidines were about 200–300-fold more potent than (−)-nicotine in PC-12 cells (Fig. 5A). The response was effectively blocked by both *d*-tubocurarine and mecamylamine, demonstrating the involvement of a ganglionic-type nicotinic receptor (Fig. 6A). β-Dihydroerythroidine was relatively weak

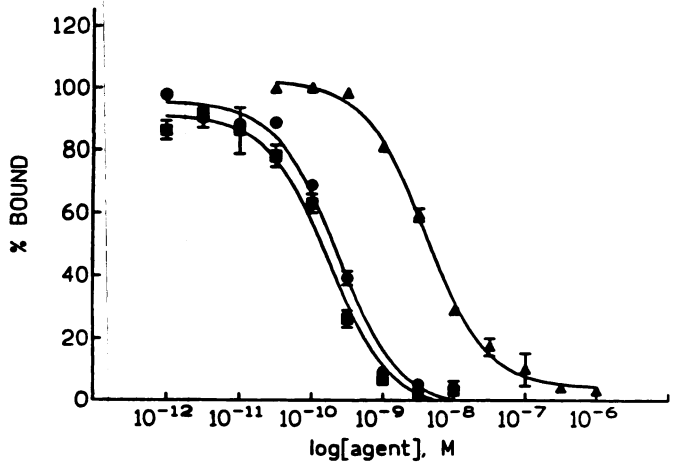


Fig. 4. Inhibition of [^{3}H]nicotine binding to rat brain membranes by epibatidines and nicotine. Binding assays were as described in Materials and Methods, with (−)-epibatidine (●), (+)-epibatidine (■), and (−)-nicotine (▲). Each value is reported as a percentage of specific binding of [^{3}H]nicotine in the absence of competing ligand and is mean ± standard error (three experiments).

TABLE 1

Inhibition of binding of [^{3}H]nicotine to rat brain membranes

Assays were as described in Materials and Methods. Values are means ± standard errors (three experiments).

Alkaloid	Pyridyl substituent	K_i
		nM
(−)-Epibatidine (1a)	Cl	0.058 ± 0.007
(+)-Epibatidine (1b)	Cl	0.045 ± 0.004
Analog 2	H	0.031 ± 0.002
Analog 3	CH_3	0.13 ± 0.02
Analog 4	I	0.48 ± 0.07
(−)-Nicotine		1.01 ± 0.09

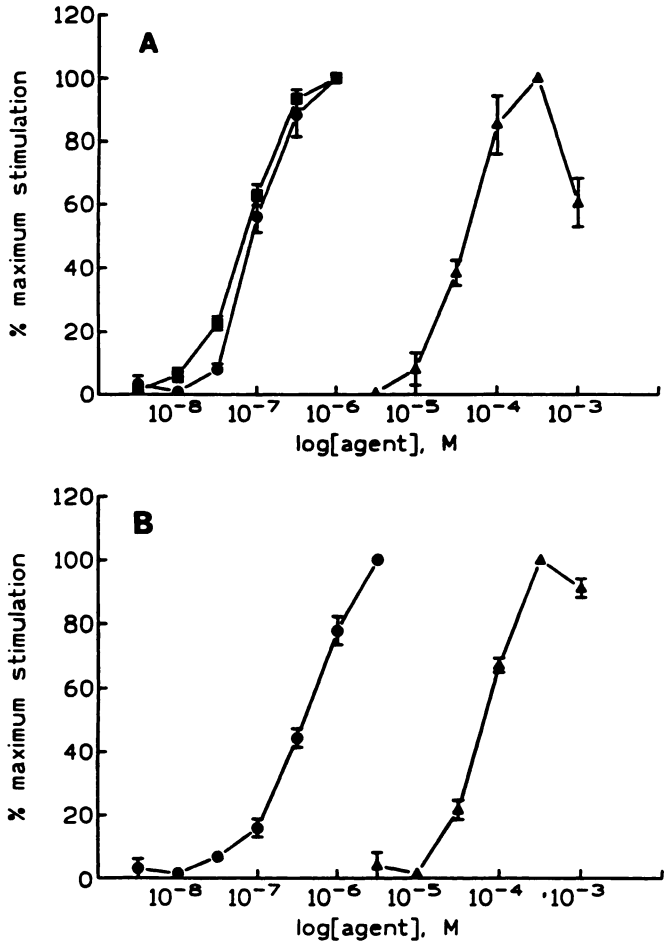


Fig. 5. Stimulation of influx of sodium by epibatidines and nicotine in rat pheochromocytoma PC-12 cells (A) and human medulloblastoma TE671 cells (B). Influx assays with ^{22}NaCl were as described in Materials and Methods, with (−)-epibatidine (●), (+)-epibatidine (■), and (−)-nicotine (▲). Each value is reported as a percentage of the maximal stimulation obtained with (−)-nicotine and is mean ± standard error (three experiments).

as an antagonist. In medulloblastoma TE671 cells, containing a central nicotinic receptor with properties similar to those of muscle-type nicotinic receptors (15), (−)-epibatidine was 5-fold less potent, with an EC_{50} value of 528 nM (Fig. 5B). (−)-Epibatidine was about 110-fold more potent than (−)-nicotine in TE671 cells. The response was effectively blocked by *d*-tubocurarine, whereas both mecamylamine and β-dihydroerythroidine were relatively weak (Fig. 6B). Electrophysiological characterization of the agonist properties of epibatidines at central and peripheral nicotinic receptors may reveal further selectivity and may provide insights into the central nicotinic receptors and pathways involved in the analgetic activity of epibatidine. The potent blockade of the analgetic activity of epibatidine by mecamylamine suggests that ganglionic-type central receptors may be involved.

Analgetic activity of a nicotinic agonist is not unprecedented. The analgetic activity of nicotine has been documented in many species (for a review, see Ref. 16). In mice, an ED_{50} of 2 mg/kg (subcutaneously) was reported (17). In contrast, in NIH Swiss strain mice, in which the epibatidines exhibit maximal analgetic effects at 0.5 μg/kg (Fig. 2), nicotine at doses of 0.1 and 3 mg/kg (intraperitoneally) evoked no significant analgesia (data not shown). There has been a history of nonresponders with respect to nicotine-elicited analgesia (see Ref. 18).

The pathways involved in nicotine-invoked analgesia have been probed using various receptor antagonists. Mecamylamine, a ganglionic nicotinic antagonist and a general noncompetitive nicotinic antagonist, does antagonize nicotine-induced analgesia (19), indicating the involvement of nicotinic receptors. Mecamylamine also blocked epibatidine-induced analgesia (Fig. 3A). The nicotinic antagonist β-dihydroerythroidine had little effect on epibatidine-induced analgesia at 2 mg/kg (Fig. 3A) and could not be tested at higher doses because of toxicity. β-Dihydroerythroidine has high activity at some central nicotinic receptors and relatively low activity at others (20).

A variety of studies suggest that nicotine-invoked analgesia involves activation, via calcium channel-dependent mechanisms, of adrenergic, serotonergic, and cholinergic pathways (see Refs. 21–23 and references cited therein). Nicotinic agonists *in vitro* do elicit release of a variety of neurotransmitters, including acetylcholine, dopamine, and serotonin, in the striatum (24–26). Epibatidine, with its high potency as an analgetic, provides a powerful tool for the delineation of pathways subserving nicotinic receptor-mediated analgesia.

Further studies of structure-activity relationships for epibatidine analogs as analgetic agents and as nicotinic agonists, when such analogs become available, should provide additional insights into the role of nicotinic receptors in the analgetic

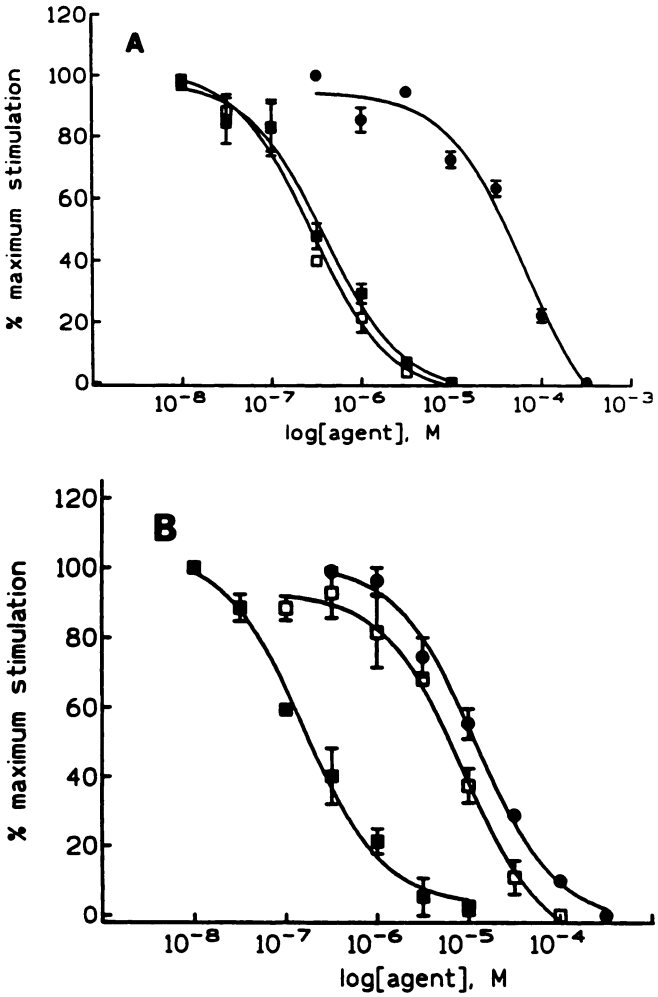


Fig. 6. Inhibition of (−)-epibatidine-elicited influx of sodium by nicotinic antagonists in rat pheochromocytoma cells (A) and human medulloblastoma TE671 cells (B). Influx assays with ^{22}NaCl were as described in Materials and Methods, with 300 nM (−)-epibatidine and indicated concentrations of *d*-tubocurarine (■), β-dihydroerythroidine (●), and mecamylamine (□). Each value is mean ± standard error (three experiments).

TABLE 2

Inhibition of (−)-epibatidine-elicited flux of sodium-22 in cultured cells

Assays were with 300 nM (−)-epibatidine, as described in Materials and Methods. Each value is mean ± standard error (three experiments).

Agent	IC_{50}	
	Pheochromocytoma PC-12 cells	Medulloblastoma TE671 cells
	μM	
d-Tubocurarine	0.38 ± 0.09	0.16 ± 0.03
β-Dihydroerythroidine	64 ± 5	11.9 ± 1.7
Mecamylamine	0.27 ± 0.04	12.5 ± 2.6

effects of epibatidine and the "toxic" effects, such as Straub tail, seen at higher concentrations (1, 2, 5). Three analogs have now been tested for affinity at central nicotine binding sites, but sufficient material was not available for *in vivo* studies. Replacement of the chloro substituent on the pyridyl ring with hydrogen had little effect on affinity (Table 1) and, thus, the chloro substituent is not necessary for nicotinic activity. However, replacement with methyl or iodo substituents resulted in about 2-fold and 10-fold reductions in affinity, respectively. The effects on activity of structural alterations in the azabicycloheptane ring or in the sterorelationship of the pyridyl ring to the azabicycloheptane ring are unknown, because such compounds have not yet been synthesized.

Acknowledgments

The authors are indebted to Dr. Teck-Peng Loh and Prof. E. J. Corey (Harvard University) for samples of (+)- and (−)-epibatidine and the three analogs and to Dr. R. Baker (Merck Sharp and Dohme Research Laboratories) for samples of epibatidine oxalates and for determination of the absolute configuration of natural epibatidine. The authors also wish to thank Dr. Arthur Jacobson and colleagues (National Institutes of Health) for assistance with establishing the analgetic assay. The NOVA Screen was conducted through the National Institute of Mental Health Drug Discovery Program administered by Dr. Jerry Cott.

References

1. Spande, T. F., H. M. Garraffo, M. W. Edwards, H. J. C. Yeh, L. Pannell, and J. W. Daly. Epibatidine: a novel (chloropyridyl)azabicycloheptane with potent analgesic activity from an Ecuadoran poison frog. *J. Am. Chem. Soc.* **114**:3475–3478 (1992).
2. Corey, E. J., T.-P. Loh, S. AchynthaRao, D. C. Daley, and S. Sarshar. Stereocontrolled total synthesis of (+)- and (−)-epibatidine. *J. Org. Chem.* **58**:5600–5602 (1993).
3. Fletcher, S. R., R. Baker, M. S. Chambers, S. C. Hobbs, and P. J. Mitchell. The synthesis of (+)- and (−)-epibatidine. *J. Chem. Soc. Chem. Commun.* **15**:1216–1218 (1993).
4. Huang, D. F., and T. Y. Shen. A versatile total synthesis of epibatidine and analogs. *Tetrahedron Lett* **34**:4477–4480 (1993).
5. Broka, C. A. Total synthesis of epibatidine. *Tetrahedron Lett.* **34**:3251–3254 (1993).
6. Clayton, S. C., and A. C. Regan. A total synthesis of (±)-epibatidine. *Tetrahedron Lett.* **34**:7493–7496 (1993).
7. Eddy, N. B., and D. Leimbach. Synthetic analgesics. II. Dithienyl-, butenyl- and dithienylbutylamines. *J. Pharmacol. Exp. Ther.* **107**:385–393 (1953).
8. Martino-Garrows, A. M., and K. J. Kellar. [^{3}H]Acetylcholine and [^{3}H](−)-nicotine label the same recognition site in rat brain. *Mol. Pharmacol.* **31**:169–174 (1986).
9. Marks, M. J., J. A. Stitzel, E. Romm, J. M. Wehner, and A. C. Collins. Nicotine binding sites in rat and mouse brain: comparison of acetylcholine, nicotine, and α-bungarotoxin. *Mol. Pharmacol.* **30**:427–436 (1986).
10. Zhang, X., and A. Nordberg. The competition of (−)-[^{3}H]nicotine binding by enantiomers of nicotine, nornicotine and anatoxin-A in membranes and solubilized preparations of different brain regions of rat. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **348**:28–34 (1993).
11. Erspamer, V., P. Melchiorri, G. Falconieri-Erspamer, L. Negri, R. Corsi, C. Severini, D. Barra, M. Simmaco, and G. Kreil. Deltorphins: a family of naturally occurring peptides with high affinity and selectivity for δ opioid binding sites. *Proc. Natl. Acad. Sci. USA* **86**:5188–5192 (1989).
12. Pedigo, N. W., H. I. Yamamura, and D. L. Nelson. Discrimination of multiple [^{3}H]5-hydroxytryptamine binding sites by the neuroleptic spiperone in rat brain. *J. Neurochem.* **36**:220–226 (1981).
13. Yamamura, H. I., and S. H. Snyder. Muscarinic cholinergic binding in rat brain. *Proc. Natl. Acad. Sci. USA* **71**:1725–1729 (1974).
14. Daly, J. W., Y. Nishizawa, M. W. Edwards, J. A. Waters, and R. S. Aronstam. Nicotinic receptor-elicited sodium flux in rat pheochromocytoma PC-12 cells: effects of agonists, antagonists and noncompetitive blockers. *Neurochem. Res.* **16**:489–500 (1991).
15. Lukas, R. J. Pharmacological distinctions between functional nicotinic acetylcholine receptors on the PC-12 rat pheochromocytoma and the TE671 human medulloblastoma. *J. Pharmacol. Exp. Ther.* **251**:175–182 (1989).
16. Pert, A. Cholinergic and catecholaminergic modulation of nociceptive reactions, in *Pain and Headache, Vol. 9, Neurotransmitters and Pain Control* (H. Akil and J. W. Lewis, eds.). S. Karger, Basel, 1–63 (1987).
17. Tripathi, H. L., B. R. Martin, and M. D. Aceto. Nicotine-induced antinociception in rats and mice: correlation with nicotine brain levels. *J. Pharmacol. Exp. Ther.* **221**:91–96 (1982).
18. Block, R. C., C. W. Y. Chin, W.-H. Wu, and V. K. Zbuzok. Nicotine-induced analgesia in rats: the role of calcium and the diversity of responders and nonresponders. *Life Sci.* **53**:PL195–PL200 (1993).
19. Martin, T. J., J. Suchocki, E. L. May, and B. R. Martin. Pharmacological evaluation of the antagonism of nicotine's central effects by mecamylamine and pempidine. *J. Pharmacol. Exp. Ther.* **254**:45–51 (1990).
20. Alkondon, M., and E. X. Albuquerque. Diversity of nicotinic acetylcholine receptors in rat hippocampal neurons. 1. Pharmacological and functional evidence for distinct structural subtypes. *J. Pharmacol. Exp. Ther.* **265**:1455–1473 (1993).
21. Iwamoto, E. T., and L. Marion. Adrenergic, serotonergic and cholinergic components of nicotinic antinociception in rats. *J. Pharmacol. Exp. Ther.* **263**:777–789 (1993).
22. Damaj, M. I., S. P. Welch, and B. R. Martin. Involvement of calcium and L-

type channels in nicotine-induced antinociception. *J. Pharmacol. Exp. Ther.* **266**:1330–1338 (1993).
23. Sahley, T. L., and G. G. Bernston. Antinociceptive effects of central and systemic administrations of nicotine in the rat. *Pyschopharmacology* **65**:279–283 (1979).
24. Rowell, P. P., and D. L. Winkler. Nicotinic stimulation of [^{3}H]acetylcholine release from mouse cerebral cortical synaptosomes. *J. Neurochem.* **43**:1593–1598 (1984).
25. Westfall, T. C., H. Grant, and H. Perry. Release of dopamine and 5-hydroxytryptamine from rat striatal slices following activation of nicotinic cholinergic receptors. *Gen. Pharmacol.* **14**:321–325 (1983).
26. Wonnacott, S., J. Irons, C. Rapier, B. Thorne, and G. G. Lunt. Presynaptic modulation of transmitter release by nicotinic receptors. *Prog. Brain Res.* **79**:157–163 (1989).

Send reprint requests to: John Daly, Building 8, Room 1A17, National Institutes of Health, Bethesda, MD 20892.