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Antinociceptive Responses to Nicotinic Acetylcholine Receptor Ligands after Systemic and Intrathecal Administration in Mice[1]

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ABSTRACT

The objective of this study was to determine which nicotinic receptor subtypes are involved in antinociception and their site of action. For that, the antinociceptive effects of several nicotinic receptor ligands were evaluated in the tail-flick test both after s.c. and intrathecal (i.t.) administration. Nicotine and other nicotine agonists increased tail-flick latencies in a dose-dependent manner after both routes of administration. Epibatidine enantiomers were the most potent agonists examined. Cytisine, a potent nicotinic ligand, failed to elicit antinociception when injected either i.t. or s.c. Despite some similarities in the effects of nicotinic agonists after i.t. and s.c. injections, their rank-order potency was different. In contrast to the s.c. results, the stereoselectivity of nicotine's effect after i.t. administration was minimal. When various nicotinic antagonists were compared after i.t. and s.c. administration, the results showed that mecamylamine and dihydro-β-erythroidine differ in potency and their degree of antagonism of some of the nicotinic agonists given i.t. These data suggest that different subtypes of nicotinic receptors may exist in the spinal cord. A good correlation was found between binding affinity to [^{3}H]-nicotine binding sites and analgesic potency after i.t. (r = 0.82), suggesting the involvement of $\alpha_4\beta_2$ receptor subunits. In contrast, studies with MLA and α-BGTX suggested a minimal role for α-BGTX-sensitive receptors in the antinociceptive effect of nicotinic agonists.

Activation of cholinergic pathways by nicotine elicit antinociceptive effects in a variety of species (Aceto *et al.*, 1986; Mattila *et al.*, 1968; Phan *et al.*, 1973). Although nicotine's effect may not extend to all types of pain and appears to be dependent on the mode of administration, recent observations suggest that cigarette smoking and nicotine reduce pain in humans (Lane *et al.*, 1995; Perkins *et al.*, 1994; Rau *et al.*, 1993) implicating a true analgesic component. Several research reports suggest that there may be more than one site of action for nicotine. For example, antinociception has been reported after systemic (Aceto *et al.*, 1986; Rogers and Iwamoto, 1993; Sahley and Berntson, 1979; Tripathi *et al.*, 1982), intracerebroventricular (Aceto *et al.*, 1986; Iwamoto, 1989; Molinero and Del Rio, 1987; Phan *et al.*, 1973; Rao *et al.*, 1996; Sahley and Berntson, 1979) and spinal (Aceto *et al.*, 1986; Christensen and Smith, 1990; Damaj *et al.*, 1995a, 1996b) administration of nicotine in rodents. However, Rogers and Iwamoto (1993) and Yakh *et al.* (1985) failed to show an antinociceptive effect after intrathecal injection of nicotine in rats.

Most evidence implicates central pathways in the action of nicotine. Indeed, systemically administered quaternary derivatives of nicotine, which do not readily penetrate the CNS, do not induce antinociception (Aceto *et al.*, 1983). In addition, antagonism of the effect of nicotine is achieved by the centrally and peripherally active antagonist, mecamylamine, but not by the quaternary antagonist, hexamethonium, which poorly crosses the blood-brain barrier (Molinero and Del Rio, 1987; Sahley and Berntson, 1979). In contrast to the reports cited above, application of nicotine via the fourth ventricle was shown to induce hyperalgesia in anesthetized decerebrate (Sloan *et al.*, 1988) and conscious rats (Hamann and Martin, 1992; Parvini *et al.*, 1993) with a possible locus of action at the dorsal posterior mesencephalic tegmentum.

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ABBREVIATIONS: nAChR, Acetylcholine nicotinic receptor; CNS, central nervous system; %MPE, maximum possible effect; CL, confidence limit; i.t., intrathecal; s.c., subcutaneous injection; ED_{50}, effective dose 50%;.AD_{50}, antagonist dose 50%; (+)-Bridge-nicotine, (+)-BN; α-Bungarotoxin, α-BGTX; dimethylphenylpiperazinium iodide, DMPP; methyllycaconitine, MLA; N-methylcarbamylcholine, N-MCC; AMP-MP, 3-(N-methyl-N-n-propylaminomethyl)pyridine; AMP-ME, 3-(N-ethyl-N-n-methylaminomethyl)pyridine; N-MNP, 1, 2, 3, 4,-tetrahydro-N-methyl)-1,6-naphhyridine.

Thus, nicotine appears to elicit both nociceptive and antinociceptive responses, perhaps reflecting the multiplicity of mechanisms involved in the effects of nicotine in the CNS. Diversity of neuronal nicotinic receptors reported recently (for review see McGeehee and Role, 1995; Sargent, 1993), may underlie such multiplicity of action which may confound pharmacological effects. In light of these observations, it is necessary to evaluate nicotinic receptor agonists after various routes of administration.

Based on autoradiography and binding studies, three classes of nicotinic receptors have been identified in the CNS (Clarke *et al.*, 1985; Schulz *et al.*, 1991). A class of binding sites with high affinity for nicotine and are labeled by [^{3}H]-nicotine, sites with high affinity for α-BGTX but low (micromolar) affinity for nicotine, and sites that display marked selectivity for neuronal bungarotoxin. Immunoprecipitation experiments indicate that $\alpha_4\beta_2$, the predominant subunit combination in the mammalian CNS, constitutes the vast majority of [^{3}H]-nicotine binding sites (Schoepfer *et al.*, 1990). However, the α_7 subunit comprises most of the high affinity [^{125}I]- α-BGTX-sensitive nAChR subtype (Seguela *et al.*, 1993). The role of these different receptor subtypes in nociceptive processes is not clearly defined.

In our study, the role of nAChRs subtypes in mediating the antinociceptive responses after systemic (s.c.) and spinal (i.t.) administration in animals was examined. The spinal cord was studied because of its involvement in the antinociceptive action of nicotine (Aceto *et al.*, 1986). For this purpose, several nicotinic ligands with a wide range of affinity to [^{3}H]-nicotine sites, were administered s.c. and i.t. to conscious mice and antinociceptive responses were measured using the tail-flick test. In addition, to delineate the role of α-BGTX-sensitive nicotinic receptors in nicotine-induced antinociception, MLA and α-BGTX, α_7 antagonists (Ward *et al.*, 1990), were used in combination with nicotinic agonists. Our studies reveal that the neuronal nicotinic receptors stimulated in the spinal cord may be distinct from those found in the brain. Antagonist specificity shows that multiple nicotinic receptors in the spinal cord may be involved in eliciting nicotine's effect in the tail-flick test.

Materials and Methods

Animals. Male ICR mice (20–25 g) obtained from Harlan Laboratories (Indianapolis, IN) were used throughout the study. The mice were housed in groups of six and had free access to food and water.

Drugs. [^{3}H](-)-Nicotine (80 Ci/mmol) was purchased from New England Nuclear (Boston, MA). (+)-and (-)-Epibatidine (hemi oxalate salt) were supplied by Dr. S. Fletcher (Merck Sharp and Dohme & Co, Essex, UK); mecamylamine hydrochloride was supplied as a gift from Merck, Sharp and Dohme & Co. (West Point, PA). Cotinine was supplied by Dr. Edward Bowman (Virginia Commonwealth University, Richmond, VA). Anabasine, cytisine and DMPP were purchased from Sigma Chemical Company (St. Louis, MO); lobeline, dihydro-β-erythroidine, N-MCC, MLA citrate and α-BGTX were purchased from RBI (Natick, MA). Nicotine enantiomers were synthesized and converted to the ditartrate salt as described by (Aceto *et al.*, 1979). Other drugs were synthesized as follows: ABT-418 HCl [(S)-3-methyl-5-(1-methyl-2-pyrrolidinyl)isoxazole)] (Garvey *et al.*, 1994), (+)-BN [(+)-*cis*-2,3,3a,4,5,9b-hexahydro-1-methyl-1*H*-pyrrolo-[3,2-*h*]isoquinoline] (Glassco *et al.*, 1993), (±)-*nor*-nicotine (Glassco *et al.*, 1994a), 6-chloronicotine (Dukat *et al.*, 1996), (±)-*iso*-nicotine (Glassco *et al.*, 1994b), AMP-MP [3-(N-methyl-N-n-propylaminomethyl)pyridine] and AMP-ME [3-(N-ethyl-N-n-methylaminomethyl)pyridine] (Glennon *et al.*, 1993), N-MNP [1, 2, 3, 4,-tetrahydro-N-methyl)-1,6-naphhyridine] (Dukat *et al.*, 1996). All drugs were dissolved in physiological saline (0.9% sodium chloride) and given in a total volume of 1 ml/100 g body weight for s.c. injections. All doses are expressed as the free base of the drug.

Intrathecal injections. Intrathecal injections were performed free-hand between the L5 and L6 lumbar space in unanesthetized male mice according to the method of Hylden and Wilcox (1980). The injection was performed using a 30-gauge needle attached to a glass microsyringe. The injection volume in all cases was 5 μl. The accurate placement of the needle was evidenced by a quick "flick" of the mouse's tail. In protocols where two sequential injections were required in an animal, the flicking motion of the tail could be elicited with the subsequent injection.

Antinociceptive assay. Antinociception was assessed by the tail-flick method of D'Amour and Smith (1941) as modified by Dewey *et al.* (1970). A control response (2–4 sec) was determined for each animal before treatment, and a test latency was determined after drug administration. To minimize tissue damage, a maximum latency of 10 sec was imposed. Antinociceptive response was calculated as %MPE, where %MPE = [(test-control)/(10-control)] $\times$ 100. Groups of 8 to 12 animals were used for each dose and for each treatment. The mice were tested 5 min after either s.c. or i.t. injections of nicotinic ligands for the dose-response evaluation. Antagonism studies were carried out by pretreating the mice i.t. with either saline or nicotinic antagonists 5 min before nicotinic agonists. The animals were tested 5 min after administration of the agonist.

[^{3}H](-)-Nicotine binding *in vitro*. [^{3}H](-)-Nicotine binding assays in rat brain were performed *in vitro* according to the method of Scimeca and Martin (1988) with minor modifications. Tissue homogenate was prepared from whole rat brain (minus cerebellum) in 10 volumes of ice-cold 0.05 M Na-K phosphate buffer (pH 7.4) and centrifuged (17,500 $\times$ *g*, 4°C) for 30 min. The pellet was then resuspended in 20 volumes of ice cold glass-distilled water and allowed to remain on ice for 60 min before being centrifuged as before. The resulting pellet was then resuspended to a final tissue concentration of 10 mg/ml of buffer. Aliquots (0.2 ml) of this final suspension were incubated at 4°C for 2 hr with phosphate buffer and [^{3}H]-nicotine (1.5 ng) in a total volume of 1 ml. Nonspecific binding was determined in the presence of 100 μM unlabeled nicotine. The incubation was terminated by rapid filtration through a Whatman GF/C glass fiber filter (presoaked overnight in 0.1% poly-L-lysine to reduce radioligand binding to the filters). Filters were washed twice with 3 ml of the buffer, and radioactivity on the filters was measured using a liquid scintillation spectrometer. Displacement of 1.5 nM [^{3}H]-nicotine binding was determined in the presence of increasing concentrations of nicotinic ligands.

Statistical analysis. Data were analyzed statistically by an analysis of variance followed by the Fisher PLSD multiple comparison test. The null hypothesis was rejected at the 0.05 level. ED_{50} and AD_{50} values with 95% CL for antinociception data were calculated by unweighted least-squares linear regression for log-doses *vs*. probits, as described by Tallarida and Murray (1987). Test for parallelism of different dose-response curves were determined as described by Tallarida and Murray (1987).

Results

Binding affinity of nicotinic ligands. The Scatchard analysis of saturation experiments with [^{3}H]-nicotine provided a kDa of 1.3 ± .08 nM and B_{max} of 253 ± 56 fmol/mg protein. The K_i values of the different nicotinic ligands are presented in table 1. Epibatidine's enantiomers, cytisine, N-MCC and 6-chloronicotine were the most potent inhibitors of the binding of [^{3}H]-nicotine. (-)-Nicotine, lobeline and ABT-418 displayed nearly equal affinity for [^{3}H]-nicotine binding sites. The binding of nicotine was stereoselective since its

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TABLE 1
Comparison of the pharmacological potencies of nicotinic ligands in the tail-flick test after s.c. and i.t. administration to their binding affinities to [^{3}H]-nicotine-labeled sites in the brain

Drug	Brain K_i (nM)	ED_{50} s.c. (μmol/kg)	ED_{50} i.t. (nmol/mouse)
(−)-Epibatidine	0.05	0.030	0.47
(+)-Epibatidine	0.045	0.033	0.67
6-Chloronicotine	0.63[a]	0.68[a]	3.37
N-MCC	0.14	20% @ 35	13.7
Lobeline	4.4	22% @ 59	23
(±)-*Iso*-nicotine	12.0[b]	27.7[b]	43
AMP-ME	28.3[c]	81.2[c]	48.5
(−)-Nicotine	1.4	8	74
N-MNP	18[c]	95[c]	80
(±)-*nor*-Nicotine	25	56	146
(+)-Nicotine	31	54.3	159
AMP-MP	1140[c]	11% @ 295	173
ABT-418	6	14	200
Anabasine	53	47.5	216
DMPP	24	3% @ 94	236
(+)-BN	>10,000	7.13	290
Cytisine	0.14	35% @ 42	10% @ 1000
Cotinine	>10,000	5% @ 100	2% @ 1000

[a] Dukat *et al.*, 1996.
[b] Glassco *et al.*, 1994b.
[c] Damaj *et al.*, 1996a.

(+)-enantiomer had almost 30-times less affinity than the (-)-enantiomer. *nor*-Nicotine (a nicotine metabolite), anabasine, AMP-ME, N-MNP and DMPP were found to have reasonable affinities with K_i values around 20 to 50 nM. Cotinine (a major nicotine metabolite) and (+)-BN at 10 μM concentrations did not displace [^{3}H]-nicotine binding. Of the nicotinic antagonists tested, only dihydro-β-erythroidine effectively inhibited [^{3}H]-nicotine binding with a K_i value of 15 ± 4.5 nM. Mecamylamine and α-BGTX at 10 μM concentrations did not displace [^{3}H]-nicotine binding. MLA, however, competed with a K_i value of 500 ± 125 nM.

Antinociceptive responses after s.c. administration. Nicotine and other nicotine agonists given s.c. increased tail-flick latencies in a dose-dependent manner (fig. 1). The (+)-enantiomer of nicotine also increased tail-flick latencies with a decreased potency (ED_{50} = 54.3 μmol/kg) compared to (-)-nicotine (ED_{50} = 8.0 μmol/kg). Table 1 summarizes the pharmacological potency of different nicotinic ligands in the tail-flick test after either s.c. or i.t. administration, along with their binding affinity to [^{3}H]-nicotine sites in the brain. Epibatidine's enantiomers and 6-chloronicotine were the most potent in the tail-flick test after s.c. injection. However, s.c. administration of AMP-MP, cotinine, DMPP and N-MCC elicited minimal responses in the tail-flick test 5 min after injection (fig. 1). In addition, cytisine and lobeline elicited partial antinociceptive effects with a response of 35 and 22%, respectively after s.c. injection. It was not possible to obtain complete dose-response curves due to the lethality and toxicity of higher doses of these drugs. No significant deviation from parallelism among the different dose-response functions after s.c. injection was found. Pretreatment with mecamylamine at a dose of 1 mg/kg, blocked nicotine-induced antinociception (fig. 2). Similar to nicotine, the antinociceptive effect of epibatidine enantiomers, (+)-nicotine, anabasine, ABT-418, (±)-*nor*-nicotine, 6-chloronicotine were blocked by mecamylamine (fig. 2). However, (+)-BN-induced antinociception was mecamylamine-insensitive (fig. 2).

Antinociceptive responses after i.t. administration. Nicotine given i.t. increased tail-flick latencies in a dose-dependent manner similar to that obtained after s.c. injection (fig. 3). Similar to nicotine, other nicotinic agonists also produced antinociception in a dose-dependent fashion after i.t. administration (fig. 3). No significant deviation from parallelism among the different dose-response functions after i.t. injection was found. In contrast to the s.c. results, the enantioselectivity of nicotine's effect after i.t. administration was not so evident. Indeed, (+)-nicotine was only two times less potent than (-)-nicotine after i.t. injection, compared to a difference of 7-fold after s.c. administration. Furthermore, as with s.c. administration, no significant enantioselectivity for epibatidine's effects was found after i.t. injection. Despite some similarities in the effects of nicotinic agonists after i.t. and s.c. injections, rank-order potency after i.t. injection is different than that observed after s.c. injection. Lobeline, almost inactive after s.c. injection, was three times more potent than nicotine in inducing antinociception after spinal administration. Similarly, AMP-MP, an aminomethylpyridine which showed little activity after s.c. injection, was active after i.t. injection (two times less potent than nicotine). In addition, (+)-BN, while almost equipotent to (-)-nicotine after s.c. injection, was clearly four times less potent after i.t.

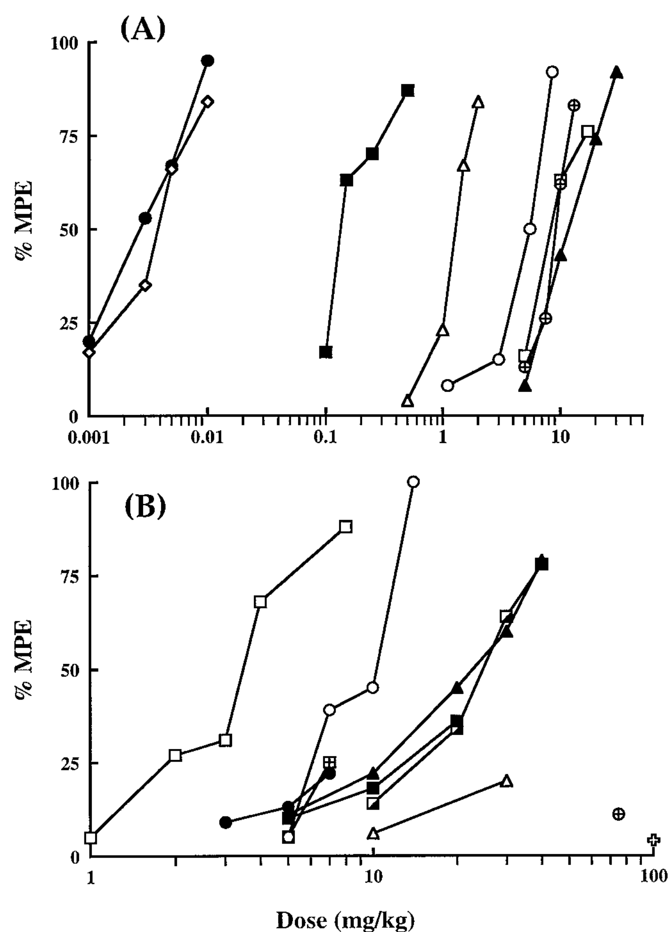

Fig. 1. Dose-response relationship of nicotinic ligands after s.c. administration in mice. (A) —△— (-)-nicotine, —○— (+)-BN, —■— 6-chloronicotine, —●— (-)-epibatidine, —◇— (+)-epibatidine, —▲— (±)-*iso*-nicotine, —⊕— (+)-nicotine. (B) —□— ABT-418, —△— DMPP, —■— lobeline, —⊕— AMP-MP, —○— anabasine, —⊞— N-MCC, —▲— WF 61, —●— cytisine, —◪— AMP-ME, —✢— cotinine. The mice were tested 5 min after drug injection in the tail-flick test. Each point represents the average %MPE for six to eight mice.

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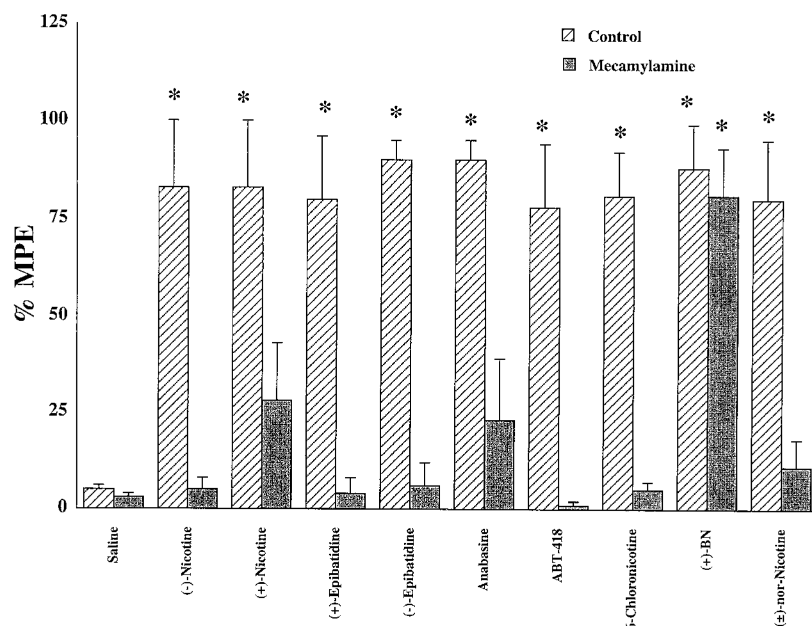

Fig. 2. Effects of mecamylamine on the antinociceptive effects of nicotinic agonists after s.c. administration. Mice were pretreated s.c. with mecamylamine (1 mg/kg) 5 min before nicotinic agonists and tested 5 min after the second injection in the tail-flick test. The nicotinic agonists were given at the following doses (ED_{84}) expressed in mg/kg: (-)-nicotine = 2; (-)-epibatidine = 0.01; (+)-epibatidine = 0.01; anabasine = 14; (+)-nicotine = 13; 6-chloronicotine = 0.5; ABT-418 = 4; (+)-BN = 6. Each point represents the average %MPE for six to eight mice. *Statistically different from saline at P < .05.

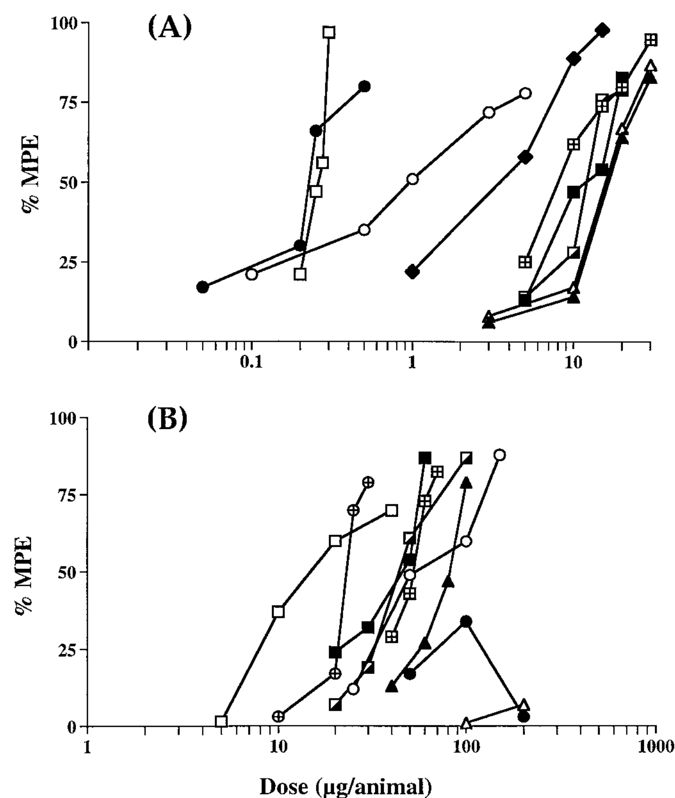

Fig. 3. Dose-response relationship of nicotinic ligands after i.t. administration in mice. (A) —△— AMP-ME, —○— 6-chloronicotine, —■— (±)-*iso*-nicotine, —●— (-)-epibatidine, —◇— (+)-epibatidine, —▲— N-MNP, —◪— (-)-nicotine, —⊞— lobeline, —◆— N-MCC. (B) —□— (+)-nicotine, —△— cotinine, —■— anabasine, —⊕— *nor*-nicotine, —○— (+)BN, —⊞— ABT-418, —▲— DMPP, —●— cytisine, —◪— AMP-MP. The mice were tested 5 min after drug injection in the tail-flick test. Each point represents the average %MPE for six to eight mice.

administration. However, (±)-*iso*-nicotine, AMP-ME and N-MNP, a conformationally constrained analog of AMP-ME, less potent than nicotine after s.c. injection, are clearly more potent after spinal administration. Contrary to what was found after s.c. injection, i.t. administration of DMPP and N-MCC elicited an antinociceptive effect in a dose-dependent manner. *nor*-Nicotine, a nicotine metabolite, seems to be more potent when given directly in the spinal cord. Furthermore, cytisine, a potent nicotinic ligand and a partial agonist after s.c. injection, failed to elicit antinociception when injected spinally.

Antagonism of the antinociceptive responses to i.t. nicotinic ligands. To further characterize ligand specificity, various nicotinic antagonists were evaluated for their ability to alter the antinociceptive effects of nicotinic agonists.

Mecamylamine. Mecamylamine, a noncompetitive nicotinic antagonist, given i.t. inhibited the antinociceptive responses of spinally given nicotine in a dose-dependent manner (fig. 4A; table 2). As illustrated, increasing doses of mecamylamine produced a gradual inhibition of the antinociceptive response to 20 μg of nicotine, with an AD_{50} of 0.8 nmol per animal. Interestingly, mecamylamine was 16 times more potent in blocking the (-)-enantiomer than the (+)-enantiomer of epibatidine. This difference was not seen with nicotine's enantiomers. In addition, nicotinic agonists differ in their sensitivity to mecamylamine (table 2). Indeed, mecamylamine blocked (+)-epibatidine, *nor*-nicotine and ABT-418 within a similar range of potency. However, 6-chloronicotine and (-)-epibatidine were more sensitive to mecamylamine. On the other hand, anabasine was much less sensitive than nicotine. In contrast to what was reported after s.c. administration (Damaj *et al.*, 1996a), mecamylamine failed to block the antinociceptive effect of N-MNP and AMP-ME after i.t. injection. Finally, mecamylamine up to a dose of 60 nmol i.t. did not significantly block the effects of lobeline, AMP-MP, (+)-BN, N-MCC and DMPP.

Dihydro-β-erythroidine. Similar to mecamylamine, dihydro-β-erythroidine, a competitive nicotinic antagonist, given i.t. inhibited the antinociceptive responses of nicotine given i.t. (fig. 4B; table 2) with an AD_{50} of 0.6 nmol/animal. The rank-order sensitivity to the blockade by dihydro-β-

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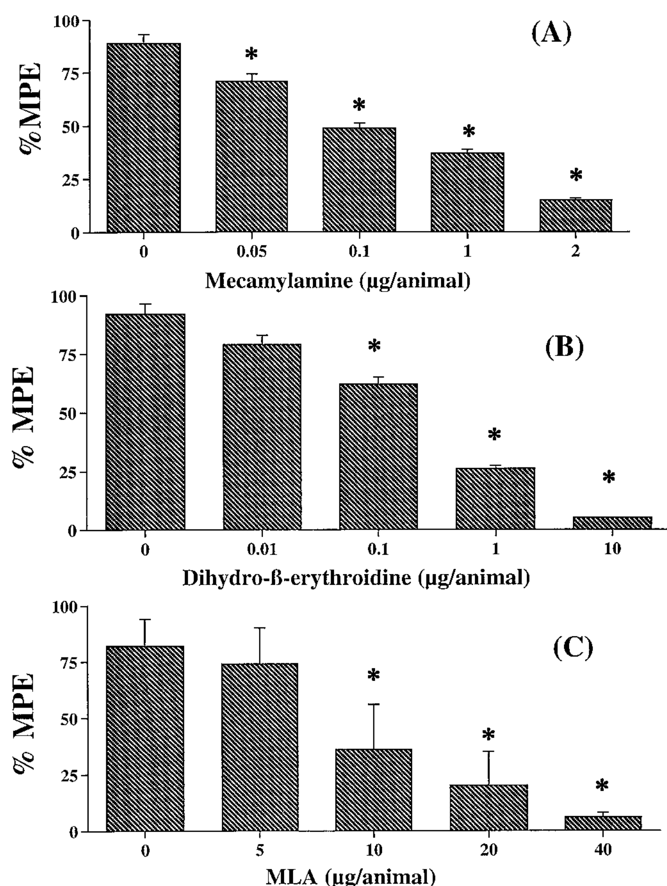

Fig. 4. Effects of (A) mecamylamine, (B) dihydro-β-erythroidine and (C) MLA on the antinociceptive effect of (-)-nicotine after i.t. administration. Mice were pretreated i.t. with different doses of nicotinic antagonists 5 min before nicotine (20 μg/animal) and tested 5 min after the second injection in the tail-flick test. Each point represents the average %MPE for six to eight mice. *Statistically different from saline (dose 0) at $P < .05$.

erythroidine was similar to that observed with mecamylamine. For example, dihydro-β-erythroidine was 21 times more potent in blocking the (-)-enantiomer than the (+)-enantiomer of epibatidine. In addition, as with mecamylamine, DMPP, N-MCC, AMP-MP, (+)-BN and lobeline were also not blocked by i.t. administration of dihydro-β-erythroidine. However, the sensitivity of 6-chloronicotine and ABT-418 to dihydro-β-erythroidine was opposite to that observed with mecamylamine. Indeed, dihydro-β-erythroidine was 180 times more potent than mecamylamine in blocking ABT-418's effect.

MLA. The plant alkaloid, MLA, produced a dose-dependent inhibition of nicotine-induced antinociception, with an AD_{50} of 16 nmol/animal (fig. 4C). MLA also significantly blocked the antinociceptive effects of (+)- and (-)-epibatidine, (+)-BN, *nor*-nicotine, (±)-*iso*-nicotine, N-MNP, AMP-MP and N-MCC in a dose-related manner (table 3). However, lobeline, AMP-ME and DMPP were not blocked by i.t. administration of MLA.

***α*-BGTX.** MLA is known to act as an antagonist at both α-BGTX binding receptors and other neuronal nicotinic receptors (Ward *et al.*, 1990). For that reason, the effects of nicotinic agonists that were MLA-sensitive were evaluated for their sensitivity to α-BGTX. When given i.t. up to a dose of 2 μg, α-BGTX failed to inhibit the antinociceptive responses to spinal nicotinic agonists (table 4). Administration (i.t.) of higher doses of α-BGTX were associated with toxicity and lethality in mice.

TABLE 2
Effects of i.t. administration of mecamylamine and dihydro-β-erythroidine on nicotinic agonist-induced antinociception after i.t. administration in mice

Ligand	Mecamylamine	Dihydro-β-Erythroidine
	AD_{50} (nnoml/mouse)[b]	AD_{50} (nnoml/mouse)[b]
(−)-Nicotine	0.8 (0.15–5)	0.6 (0.1–2.8)
(+)-Nicotine	1.1 (0.3–3.0)	0.75 (0.3–2.0)
(−)-Epibatidine	0.15 (0.02–1.2)	0.7 (0.15–3.4)
(+)-Epibatidine	2.4 (1.4–12)	15 (4–59)
6-Chloronicotine	0.02 (0.002–0.02)	1.5 (0.4–5.1)
nor-Nicotine	0.3 (0.08–1.3)	0.72 (0.1–5.0)
(±)-*Iso*-nicotine	1.4 (0.3–3.6)	4.5 (1.1–17.6)
ABT-418	1.8 (0.6–5.0)	0.01 (0.002–0.02)
Anabasine	28 (9–45)	ND[a]
N-MNP	10% blockade @ 60	9% blockade @ 36
AMP-ME	0% blockade @ 60	0% blockade @ 36
AMP-MP	15% blockade @ 60	12% blockade @ 36
DMPP	23% blockade @ 60	0% blockade @ 36
N-MCC	11% blockade @ 60	0% blockade @ 36
Lobeline	21% blockade @ 60	0% blockade @ 36
(+)-BN	20% blockade @ 60	10% blockade @ 36

[a] ND, Not determined.
[b] Mice were pretreated i.t. with different doses of mecamylamine or dihydro-β-erythroidine 5 min before nicotine agonists and mice were tested 5 min after the administration of the agonist in the tail-flick test. Results are expressed as AD50 values (±CL).

TABLE 3
Effects of i.t. administration of MLA on nicotinic agonist-induced antinociception after i.t. administration in mice

Ligand	MLA
	AD_{50} (nnoml/mouse)[a]
(−)-Nicotine	16 (10–23)
(+)-Nicotine	5 (1.5–8)
(−)-Epibatidine	7 (2–17)
(+)-Epibatidine	5 (2–14)
6-Chloronicotine	34 (24–43)
nor-Nicotine	25 (17–28)
(+)-BN	25 (16–40)
(±)-*Iso*-nicotine	6 (4–15)
N-MCC	46 (24–89)
ABT-418	15 (8–19)
N-MNP	17 (14–23)
AMP-ME	2% blockade @ 90
AMP-MP	2% blockade @ 90
DMPP	20% blockade @ 90
Lobeline	5% blockade @ 90

[a] Mice were pretreated i.t. with different doses of MLA 5 min before nicotine agonists and mice were tested 5 min after in the tail-flick test. Results are expressed as AD50 values ± CL.

Cytisine. Cytisine, a high affinity nicotinic ligand which is known to have agonist properties in several nicotinic preparations, blocked nicotine-induced antinociception in a dose-dependent manner following i.t. injection (table 5). Cytisine also blocked the response generated by 0.2 μg of the epibatidine enantiomers. Interestingly, (-)-epibatidine seems to be more sensitive to the effect of cytisine than (+)-epibatidine. In contrast to nicotine and epibatidine, the antinociceptive effects of other nicotinic agonists were not blocked by cytisine at all doses tested.

Discussion

Little work has been done to distinguish the subtypes of nicotinic receptors involved in mediating the pharmacologi-

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TABLE 4
Effects of α-BGTX (i.t.) on nicotinic agonist-induced antinociception after i.t. administration in mice

Ligand	α-BGTX[a]	
	Control	% MPE
(−)-Nicotine	78 ± 13	69 ± 19
(−)-Epibatidine	98 ± 2	75 ± 16
(+)-Epibatidine	98 ± 2	68 ± 19
DMPP	62 ± 19	85 ± 15
N-MCC	75 ± 16	88 ± 9
(+)-Nicotine	83 ± 11	85 ± 12
nor-Nicotine	71 ± 18	82 ± 10
6-Chloronicotine	88 ± 10	90 ± 9
N-MNP	78 ± 13	83 ± 12
AMP-ME	82 ± 14	80 ± 11
ABT-418	77 ± 15	85 ± 14
(±)-*Iso*-nicotine	89 ± 7	88 ± 12
AMP-MP	90 ± 14	86 ± 14
Lobeline	76 ± 15	85 ± 15
(+)-BN	96 ± 4	90 ± 10

[a] Mice were pretreated i.t. with a doses of 2 μg per animal of α-BGTX 5 min before nicotine agonists and mice were tested 5 min after the second injection in the tail-flick test. Data are presented as mean ± S.E. of 8 to 12 animals/group.

TABLE 5
Effects of cytisine (i.t.) on nicotinic agonist-induced antinociception after i.t. administration in mice

Ligand	Control	Dose of Cytisine (i.t.)[a]			
		5 μg	20 μg	40 μg	80 μg
(−)-Nicotine	93 ± 20	75 ± 12	53 ± 23	12 ± 8	
(−)-Epibatidine	84 ± 17	33 ± 18	47 ± 17	36 ± 14	1 ± 1
(+)-Epibatidine	98 ± 2			77 ± 23	30 ± 18
(+)-Nicotine	83 ± 11				95 ± 5
nor-Nicotine	71 ± 18				76 ± 15
ABT-418	77 ± 15				76 ± 12
(+)-BN	83 ± 12				92 ± 8
N-MCC	79 ± 14			95 ± 5	98 ± 2
Lobeline	90 ± 10			98 ± 2	92 ± 8

[a] Mice were pretreated i.t. with different doses of cytisine 5 min before nicotine agonists and mice were tested 5 min after in the tail-flick test. Data are presented as mean ± S.E. of %MPE; 8 to 12 animals/group were used.

cal effects of nicotine in the different parts of the CNS. Since the molecular composition of native CNS nicotinic receptors *per se* is not known with any certainty, pharmacological approaches can be used to implicate the involvement of receptor subtypes in the actions of nicotine.

Consistent with previous reports (Martin *et al.*, 1983; Tripathi *et al.*, 1982), the s.c. injection of nicotine increased tail-flick latencies in a stereospecific and mecamylamine-sensitive manner. Mecamylamine (s.c.) almost completely blocked the effects of all active compounds in the tail-flick test except for (+)-BN, a bridge-nicotine analog that lacks affinity to [^{3}H]-nicotine binding sites (Glassco *et al.*, 1993). The fact that s.c. administration of DMPP and N-MCC, compounds that poorly penetrate the blood-brain barrier, showed little antinociceptive activity confirms previous reports that nicotinic analgesia is centrally mediated (Aceto *et al.*, 1983; Sahley and Berntson, 1979). Although s.c. administration results in simultaneous delivery of nicotine to multiple sites including the spinal cord, our results suggest that the pharmacology of nicotine differs at spinal and supraspinal sites. Additionally, the pharmacology of nicotinic ligands differs between the two routes of administration. Comparison of the rank-order potency of the different nicotinic ligands and their sensitivity to nicotinic antagonists after s.c. and i.t. administration, suggests that spinal and supraspinal nicotinic receptors may have different features. Indeed, rank-order potency after i.t. injection is different from that observed after s.c. injection. Lobeline, almost inactive after s.c. injection, is very potent in inducing antinociception after spinal administration. This difference is probably not due to a distribution factor, because lobeline is reported to penetrate the blood-brain barrier after s.c. injection (Reavill *et al.*, 1990). A similar effect was seen with AMP-MP, an aminomethylpyridine which binds with very low affinity, after i.t. administration. In addition, (+)-BN, while almost equipotent to (-)-nicotine after s.c. injection, is clearly less potent after i.t. administration. In contrast to (+)-BN, (±)-*iso*-nicotine, AMP-ME and N-MNP, which were less potent than nicotine after s.c. injection, were clearly more potent after spinal administration. In addition, *nor*-nicotine, a nicotine metabolite, seems to be more potent when given directly in the spinal cord (26 and 2 times less potent than nicotine after s.c. and i.t., respectively). Such difference in potency may reflect difference in receptor subtypes and/or function. However, the influence of pharmacokinetic factors cannot be ignored. Indeed, peak effect, distribution profile and metabolic differences after s.c. and i.t. administration can influence the potency of nicotinic agonists. We have evaluated the time-course effect of nicotinic agonists in our analgesic assays and found that they have a rapid onset of actions (maximal effects at 5 min) and very short duration (30 to 60 min after either s.c. or i.t. injection) (data not shown). Therefore, a pretreatment time of 5 min, where a maximal analgesia was observed, was used in our tests. However, distribution patterns and metabolic profiles after s.c. and i.t. administration were not investigated. Finally, cytisine that is a potent nicotinic ligand, acts as a partial agonist after s.c. injection and as an antagonist after i.t. injection. The antagonism produced by cytisine could result from a secondary effect to its role as a $\alpha_4\beta_2$ partial agonist as suggested by Papke and Heinemann (1994) or as an open-channel blocker (Luetje and Patrick, 1991).However, when receptor sensitivities to various nicotinic antagonists after i.t. and s.c. administration are compared, our results showed that mecamylamine and dihydro-β-erythroidine differ in potency and their antagonism of some of the nicotinic agonists in the mouse spinal cord. Indeed, mecamylamine which blocks (-)-nicotine with almost the same potency as dihydro-β-erythroidine after i.t. injection, is 10 times more potent when it is given s.c. (Damaj *et al.*, 1995b). Moreover, mecamylamine is more potent than dihydro-β-erythroidine in blocking the enantiomers of epibatidine, (±)-*iso*-nicotine and 6-chloronicotine in the spinal cord. However, dihydro-β-erythroidine was more potent in blocking ABT-418 than mecamylamine. The difference in potency was not only seen between the nicotinic antagonists, but agonists differed in their sensitivity to the same antagonist (see table 2). For example, since epibatidine enantiomers display similar affinities for [^{3}H]-nicotine and [^{3}H]-cytisine binding sites and demonstrate similar pharmacological effects after s.c. (Damaj *et al.*, 1994) and i.t. administration, it was expected that the enantiomers would be blocked in a similar fashion by nicotinic antagonists. However, blockade experiments with mecamylamine and dihydro-β-erythroidine revealed a differential sensitivity of the epibatidine enantiomers to these antagonists with the (-)-enantiomer being 15 to 20 times more sensitive to the blockade effect of mecamylamine and dihydro-β-erythroidine. Interestingly, such difference was

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not seen with nicotine enantiomers. Thus, taken together, these data suggest that different subtypes of nicotinic receptors may exist in the spinal cord. Our findings correlated with spinal receptors binding results reported by Khan *et al.*, (1994a), which suggest that the spinal cord and brain receptors appear to have distinct features and present differential selectivity to nicotinic ligands. The differences between these two antagonists are not unique to the spinal cord, and it has been reported in several brain areas. For example, mecamylamine (non-competitive antagonist) and dihydro-β-erythroidine (competitive antagonist) act as nicotinic antagonists in the rat hippocampus (Alkondon and Albuquerque, 1991) and medial habenula (Mulle *et al.*, 1991), whereas dihydro-β-erythroidine acts in the rat prefrontal cortex (Vidal and Changeux, 1989).

In assessing the involvement of different nicotinic receptors subunits, our data suggest that $\alpha_4\beta_2$ subunits combination are involved in nicotine-induced antinociception. Indeed, a good correlation exists between binding affinity to [^{3}H]-nicotine binding sites and analgesic potency after i.t. injection (a coefficient of 0.82) (fig. 5). However, correlating rat nicotine binding data with intrathecal mouse analgesic potencies should be done cautiously. Contradictory results has been reported after intrathecal administration of nicotine in rodents. Indeed, Aceto *et al.* (1986) and Christensen and Smith (1990) found that nicotine given i.t. in rats is active in the tail-flick (Damaj *et al.*, 1996a). Although a good correlation between rat nicotine binding affinities with s.c. mouse analgesic potencies was found (Damaj *et al.*, 1996a), such correlation may not exist with binding affinities in the mouse brain. However, the involvement of $\alpha_4\beta_2$ receptors relies mainly on the pharmacological studies with different nicotinic ligands. Furthermore, the antinociceptive effects of several nicotinic agonists tested (see tables 2 and 6) are blocked by dihydro-β-erythroidine, a competitive nicotinic antagonist. However, multiple mechanisms and subunit combinations may be also involved since, contrary to what is reported after intracerebroventricular administration (Yang and Buccafusco, 1994), i.t. DMPP and N-MCC were not blocked by dihydro-β-erythroidine and mecamylamine. In addition, other nicotinic agonists such as N-MNP, lobeline and (+)-BN, elicited an antinociceptive effect that was not blocked by the nicotinic antagonists mentioned above.

The results with MLA suggest the involvement of α_7 subunits in nicotinic analgesia. Indeed, MLA significantly blocked the effects of nicotine, epibatidine and other nicotinic ligands after i.t. injection with different potencies. MLA, which potently inhibits [^{125}I]α-BGTX binding sites (K_i = 4 nM) in contrast to its weaker interactions with other neuronal nicotinic receptors (μM range), has been classified as a competitive antagonist of α_7 nicotinic receptors (Ward *et al.*, 1990). However, the facts that relatively high doses of MLA were needed to block the effects of the nicotinic agonists and that i.t. injection of α-BGTX was completely ineffective as an antagonist in this test, would weaken the involvement of α_7 subunits in nicotine-induced antinociception but not completely exclude it. Recently, Khan *et al.* (1994b) observed that MLA administered i.t. but not α-bungarotoxin blocked the cardiovascular and behavioral effects of nicotine injected spinally. The authors suggested that MLA may antagonize a wider spectrum of neuronal nicotinic receptors at the spinal level. In addition, Rao *et al.*,(1996) showed that α-BGTX-sensitive receptors failed to block nicotine-induced antinociception after i.c.v. administration in rat. Because neither MLA nor α-bungarotoxin were able to block N-MCC and lobeline's effects, our results would suggest the involvement of other receptor subunits, such as α_3 subunits. However, limited availability of n-bungarotoxin has precluded its use in i.t. injection. The fact that cytisine which is a full β_4 agonist (Luetje and Patrick, 1991) and possess agonistic properties in several preparations, elicited a minor effect in the tail-flick test after i.t. administration, suggests that spinal β_4 subunits are probably not involved in nicotine-induced antinociception.

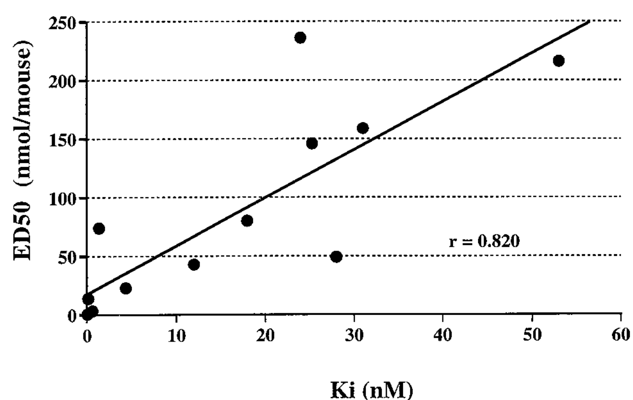

Fig. 5. Correlation between receptor affinity (K_I expressed as nM) to rat brain [^{3}H]-nicotine sites and antinociceptive potency (ED$_{50}$ values expressed as mg/kg) for nicotinic agonists after i.t. injection.

TABLE 6
Summary of the activity of differents nicotinic ligands and their sensitivity to various nicotinic antagonists in the tail-flick test

Ligand	S.C.	I.T.	MECA	DβHE	MLA	α-BGTX	CYT
(−)-Epibatidine	+	+	+	+	+	−	+
(+)-Epibatidine	+	+	+	+	+	−	+
6-Chloronicotine	+	+	+	+			ND[a]
N-MCC	−	+	−	−	+	−	−
Lobeline	−	+	−	−	−	−	−
(−)-Nicotine	+	+	+	+	+	−	+
(±)-*Nor*-nicotine	+	+	+	+	+	−	−
ABT-418	+	+	+	+	+		−
Anabasine	+	+	+	+	ND	ND	ND
DMPP	−	+	−	−	−	−	ND
(+)-Nicotine	+	+	+	+	+	−	−
(±)-*Iso*-nicotine	+	+	+	+	+	−	ND
AMP-MP	−	+	−	−		−	ND
N-MNP	+	+	−	−	+	−	ND
AMP-ME	+	+	−	−		−	ND
(+)-BN	+	+	−	−	+	−	−
Cytisine	−	−					
Cotinine	−	−					

[a] ND, Not determined.

In summary, we demonstrated that spinal and supraspinal sites appear to contribute to the antinociceptive effects of nicotinic agonists. Our studies also demonstrate the complexity involved in determining the receptor subtypes mediating the pharmacological effects of nicotine. It would appear that the mechanisms for spinal and supraspinal antinociception are not identical. These differences could be due to activation of differential neuronal pathways, involvement of multiple receptor subtypes and pharmacokinetic factors.

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