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S-(-)-ET 126 : A POTENT AND SELECTIVE M_1 ANTAGONIST IN VITRO AND IN VIVO

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Summary

The pharmacological profile of the competitive muscarinic antagonist S-(-)-α-(hydroxymethyl)benzeneacetic acid 1-methyl-4-piperidinyl ester (S-(-)-ET 126) was evaluated on M_1 (rabbit vas deferens; pA_2=8.99), M_2 (rat left atrium; pA_2=8.21) and M_3 (rat ileum; pA_2=6.84) muscarinic receptors in comparison with pirenzepine. The drug shows a subtype selectivity (M_1/M_2=8; M_1/M_3=178; M_2/M_3=22) that proposes it as a useful pharmacological tool for receptor studies. S-(-)-ET 126, like pirenzepine, prevents the antinociception induced by M_1 agonists (McN-A-343 and AF-102B). Unlike pirenzepine and spirotramine, the compound is able to cross the blood brain barrier which makes it useful for in vivo investigations.

Key Words: S-(-)-ET 126, M_1 selective muscarinic antagonist, M_1, M_2 and M_3 subtype, analgesia

Radioligand binding and functional studies with selective agonists and antagonists have demonstrated that there are at least three pharmacologically distinct muscarinic receptor subtypes (M_1-M_3) (1, 2). Antagonists used in this subclassification include pirenzepine (M_1), AF-DX 116, methoctramine, himbacine (M_2) and HHSiD, p-F-HHSiD, sila-hexocyclium and 4-DAMP (M_3) (for reviews see 2, 3). Molecular cloning tecniques have recently revealed in various animal mammalian species the existence of five distinct molecular muscarinic receptor proteins (m_1-m_5) (4, 5, 6, 7, 8). The antagonist binding properties of the cloned receptors showed a close correlation between the m_1, m_2 and m_3 gene products and the pharmacologically defined M_1, M_2 and M_3 muscarinic receptor subtypes (6, 9, 10).

It has long been known that the activation of the cholinergic system induces antinociception in laboratory animals (11, 12, 13, 14, 15, 16, 17). Bartolini et al. (18) have demonstrated that muscarinic analgesia in mice and rats is mediated by postsynaptic M_1 receptors. These authors reported that M_1 selective agonists McN-A-343 and AF-102B were able to produce a significant enhancement of the pain threshold while the M_2 selective agonist arecaidine propargil ester (APE) was not. Moreover, Bartolini et al. (18) have demonstrated that the M_1 antagonists dicyclomine and pirenzepine, contrary to the M_2 antagonist AF-DX 116, antagonized antinociception induced by both unselective (oxotremorine) and M_1 selective (McN-A-343, AF-102B) muscarinic agonists.

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In order to investigate the properties of each muscarinic receptor subtype, selective ligands are necessary. To date, compounds that are able to consistently discriminate between M_1 and M_3 muscarinic receptor subtypes are not available. Therefore we thought it important to further evaluate the pharmacological profile of the compound (S-(-)-α-(hydroxymethyl)benzene- acetic acid 1-methyl-4-piperidinyl ester) currently designed as S-(-)-ET 126 which is structurally related to atropine (Fig. 1) and has already been described (19). First, in vitro functional studies were carried out to determine S-(-)-ET 126 affinity towards rabbit vas deferens M_1 receptors (20), rat atrial M_2 and rat ileal M_3 (21) receptors. Second, in vivo antinociception experiments were performed to determine the ability of S-(-)-ET 126 to antagonize antinociception induced by M_1 agonists.

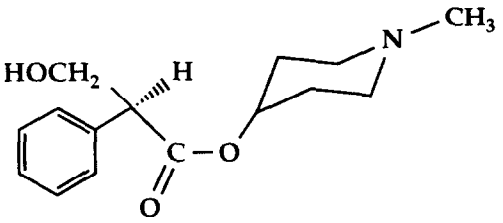

FIG.1

Steric structure of S-(-)-ET 126

Methods

In vitro functional studies

Rabbit isolated vas deferens: Experiments on isolated rabbits vas deferens were performed according to Eltze et al. (21).

Rat isolated left atria and ileum : Isolated left atria and ileum fragments were prepared according to Paton and Zar (22). Bath fluid temperature was maintained at 32°C (atria) and 37 °C (ileum), respectively. Atria were electrically stimulated (2 Hz, 3 ms, 10 V) by means of two platinum electrodes. Carbachol negative inotropic effects on isometric atria contractions and isotonic ileum contractions induced by ACh were recorded before and 1 h after perfusion with antagonists.

Determination of antagonist affinities : After a stabilization time of 30-60 min, agonist concentration-response curves were plotted before and after equilibration with antagonists. In separate control experiments no significant changes in tissue sensitivity to the agonist were observed over the period required for the determination of two concetration-response curves. Three different concentrations of each antagonist were used (log conc. interval 0.5; n= 4-6 for each conc.). The antagonists were allowed to equilibrate for 60 min. No more than two concentrations of antagonist were tested in the same preparation. Agonist EC_{50} values in the

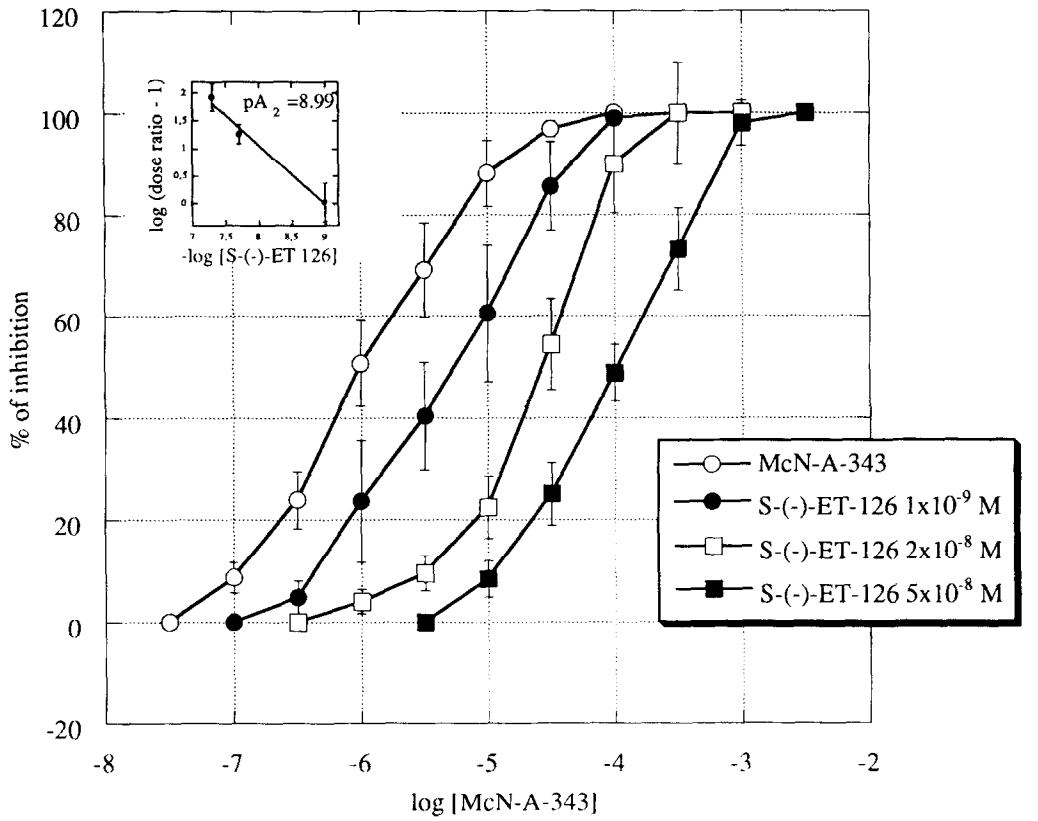

FIG. 2

Antagonism by S-(-)-ET 126 of muscarinic receptors mediating inhibition of rabbit vas deferens. Concentration-response curves for McN-A-343 in the absence (○) or in the presence of increasing concentrations (● $1x10^{-9}$; □ $2x10^{-8}$; ■ $5x10^{-8}$M) of S-(-)-ET 126.
Each point represents the mean ± s.e.m. of at least 4 experiments.
Insert: Schild analysis of antagonism of McN-A-343-induced contractions.

absence and in the presence of antagonists were determined graphically for the calculation of a dose ratios. The slope of Arunlakshana-Schild plots (23) were determinated by linear regression, according to the least-squares method. pA_2 values were estimated by fitting the best straight line to the data with a slope of unity as required by the theory (24).

In vivo antinociception experiments

Hot plate test : Male Swiss albino mice (23-30g) were placed inside a stainless steel container, thermostatically set 52.5 ± 0.1 °C in a precision water-bath from CW Mechanical Workshop, Siena, Italy. Reaction times (sec), were measured with a stopwatch before and at 15, 30, 45 and 60 min after treatment. The endpoint used was the licking of the fore or hind paws. Those mice scoring below 12 and over 18 s in the pretest were rejected. An arbitrary cut-off time of 45 s was adopted.

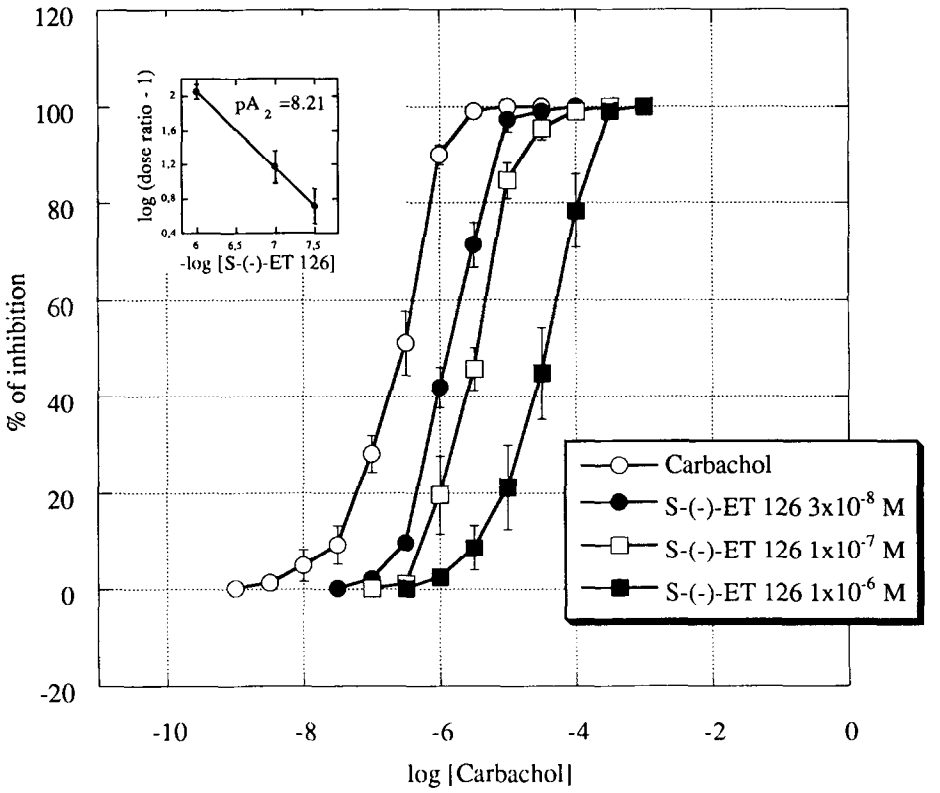

FIG. 3

Antagonistic effect of S-(-)-ET 126 on muscarinic receptors mediating inhibition of rat left atrium. Concentration-response curves for carbachol in the absence (○) or in the presence of increasing concentrations (● $3x10^{-8}$; □ $1x10^{-7}$; ■ $1x10^{-6}$ M) of S-(-)-ET 126.
Each point represents the mean ± s.e.m. of at least 4 experiments.
Insert: Schild analysis of antagonism of carbachol-induced contractions.

Data are presented as means ± s.e.m. of *n* experiments. Differences between mean values were tested for statistical significance by Student's *t* test; the level of significance chosen was $P< 0.05$.

The following drugs were used: S-(-)-ET 126 (S-(-)-α-(hydroxymethyl)benzene-acetic acid 1-methyl-4-piperidinyl ester; prepared in the Department of Pharmaceutical Sciences of Florence, Italy as previously described by Gualtieri et al. (19); the compound has an optical purity greater than 97%); acetylcholine chloride (Merck); pirenzepine dihydrochloride and McN-A-343 4-(N-[3-chlorophenyl]-carbamoyloxy)-2-butynyl-trimethylammonium chloride (R.B.I.); carbamylcholine chloride, atropine sulphate and yohimbine hydrochloride (Sigma); AF-102B (Inst. for Neurobiol. Res., Bruxelles, Belgium). Other chemicals were of the highest quality commercially available. All the drugs were dissolved in the nutritive solutions immediately before use. The concentrations given in the text are expressed as salts. Drug concentrations were prepared

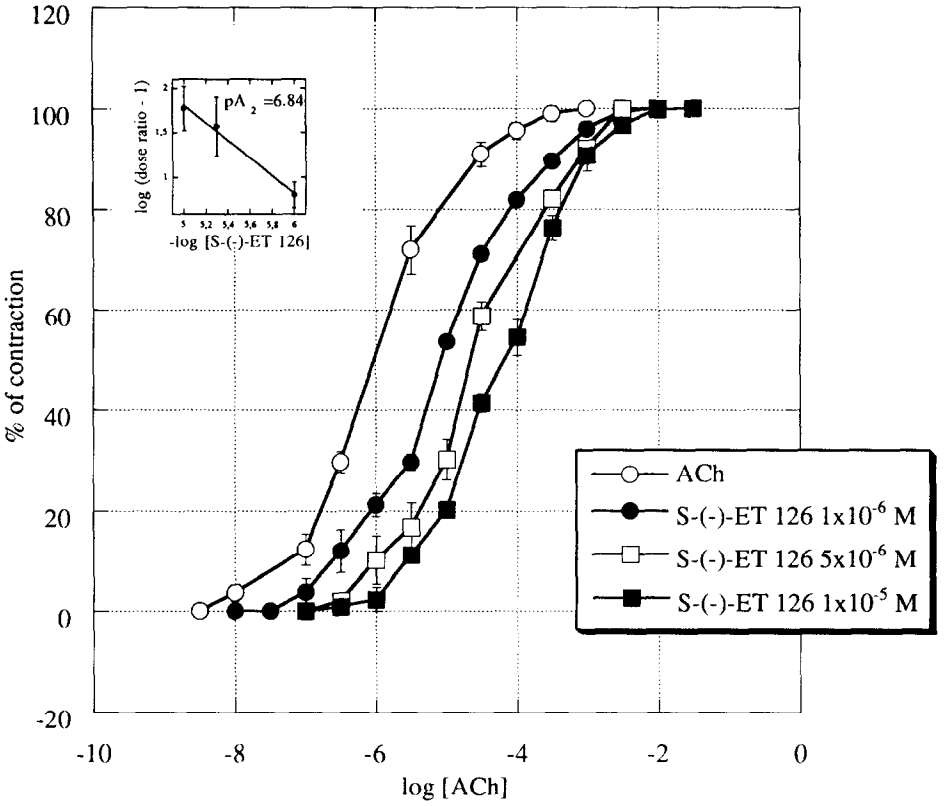

FIG. 4

Antagonistic effect of S-(-)-ET 126 on muscarinic receptors mediating contraction of rat ileum. Concentration-response curves for acetylcholine in the absence (○) or in the presence of increasing concentrations (● $1x10^{-6}$; □ $5x10^{-5}$; ■ $1x10^{-5}$ M) of S-(-)-ET 126.
Each point represents the mean ± s.e.m. of at least 4 experiments.
Insert: Schild analysis of antagonism of acetylcholine-induced contractions.

in such a way that the necessary dose could be administered in a volume of 10 ml kg^{-1} by intraperitoneal (i.p.) injection. Intracerebroventricular (i.c.v.) administration was performed under ether anaesthesia using isotonic saline as a solvent, according to the method described by Haley and McCormick (25). Briefly, during anaesthesia, mice were grasped firmly by the loose skin behind the head. A 0.4 mm external diameter, hypodermic needle attached to a 10 µl syringe was inserted perpendicularly through the skull, no more than 2 mm into the brain of the mouse, where 5 µl were then administered. The injection site was 1 mm from the midline along a line drawn through to the anterior base of the ears. To ascertain the exact point into which the drugs were administered, some mice were injected i.c.v. with 5 µl of diluited 1:10 Indian ink and their brains examined macroscopically after sectioning.

TABLE 1

Affinity profiles of S-(-)-ET 126 and pirenzepine at muscarinic M_1-receptors in rabbit vas deferens, M_2-receptors in rat left atrium and M_3-receptors in rat ileum. The ratios of affinity constants are given as a measure of receptor selectivity.

	pA_2 values			**Selectivity ratios**		
Muscarinic Antagonists	**M_1** *rabbit vas deferens*	**M_2** *rat left atrium*	**M_3** *rat ileum*	**M_1/M_2**	**M_1/M_3**	**M_2/M_3**
S-(-)-ET 126	8.99 ± 0.15	8.21 ± 0.06	6.84 ± 0.15	8	178	22
	(1.08 ± 0.13)	(0.96 ± 0.04)	(0.98 ± 0.10)			
	[9.07 ± 0.08]	[8.16 ± 0.02]	[6.82 ± 0.03]			
Pirenzepine	7.89 ± 0.28	6.47 ± 0.19	6.61 ± 0.18*	29	21	1
	(0.98 ± 0.11)	(1.00 ± 0.18)				
	[7.94 ± 0.09]	[6.47 ± 0.05]				

pA_2 values are the mean ± SEM of 12-16 preparations. *pK_B values are obtained with pirenzepine 1 μM. In parentheses is the slope of the linear regressions. In square parentheses are the pA_2 values obtained constraining the slope of the linear regressions to the unity. Selectivity ratios were calculated as antilogs of the difference between pA_2 values obtained constraining the slope of the linear regression to the unity (or pK_B values).

Results

In vitro functional studies: S-(-)-ET 126 concentration-dependently blocked the McN-A-343-induced inhibition of twitch contractions of rabbit vas deferens (Fig. 2). S-(-)-ET 126 antagonized the negative inotropic carbachol-induced effect in rat left atrium (Fig. 3) and the contractile responses to acetylcholine in rat ileum (Fig. 4). Increasing concentrations of S-(-)-ET 126 produced parallel shifts of the agonist concentration-response curves progressively to the right. No appreciable change in basal tension or maximum agonist response was observed. pA_2 values of S-(-)-ET 126 and pirenzepine, used as reference drug, are shown in Table 1. Regression lines for both compounds were not significantly different from unity (Table 1). The selectivity ratios for S-(-)-ET 126 and pirenzepine, obtained as differences between pA_2 values, are reported in Table 1.

In vivo studies: Regarding the in vivo studies, the antagonism exerted by S-(-)-ET126 (0.01 μg per mouse i.c.v.), pirenzepine (0.01 μg per mouse i.c.v.) and atropine (1 μg per mouse i.c.v.) of antinociception induced by McN-A-343 (30 μg per mouse i.c.v.) and AF-102B (5 mg kg^{-1} i.p.) in the mouse hot-plate test is reported in Fig. 5. Similar results were obtained when S-(-)-ET 126 was administered intraperitoneally thus showing that the drug is able to cross the blood brain barrier (data not shown). In the same experimental conditions S-(-)-ET 126, pirenzepine and atropine did not modify the pain threshold when given alone (Fig. 5).

Discussion

The receptor selectivity profile of the tropic acid ester S-(-)-ET 126 was evaluated by using in vitro functional studies in comparison with the reference drug pirenzepine. These data were supported by in vivo behavioral experiments.

As shown by functional studies, S-(-)-ET 126 interacts with the muscarinic receptor as a competitive antagonist. In fact, agonist concentration-response curves were parallely displaced to the right without depression of maximum responses. Moreover, the slopes of the Schild regression lines were not significantly different from unity. These studies revealed that S-(-)-ET 126 has an affinity for M_1 receptors in rabbit vas deferens ($pA_2 = 8.99$) and for M_2 receptors in rat left atrium ($pA_2 = 8.21$) that are respectively about 10- and 100-fold higher than those obtained for pirenzepine ($pA_2 = 7.89$ for M_1; $pA_2 = 6.47$ for M_2). On the other hand, S-(-)-ET 126 ($pA_2 = 6.84$) and pirenzepine ($pK_B = 6.61$) show similar pA_2 values for the M_3 receptor subtype. Regarding the capability of discriminating among the different muscarinic receptor subtypes, S-(-)-ET 126 is able to better discriminate between M_1/M_3 and between M_2/M_3 receptors than pirenzepine. S-(-)-ET 126 has, in fact, a selectivity ratio of 178 with respect to M_1/M_3 and of 22 with respect to M_2/M_3. In our hands pirenzepine exhibits selectivity ratios of 21 for M_1/M_3 and 1 for M_2/M_3 and these findings are in good accord with the selectivity reported in the literature (26). By contrast, S-(-)-ET 126 shows about a 4-time lower ability to discriminate between M_1 and M_2 receptors than pirenzepine (selectivity ratio $M_1/M_2 = 29$ for pirenzepine and 8 for S-(-)-ET 126).

In order to confirm the M_1 antagonistic properties of S-(-)-ET 126 by using in vivo behavioral studies, the ability of S-(-)-ET 126 to prevent muscarinic analgesia was evaluated. It has been in fact reported that the analgesic effect subsequent to the activation of the cholinergic system is mediated by the M_1 receptor subtype (see introduction). S-(-)-ET 126, like pirenzepine and atropine, is able to prevent analgesia induced by M_1 agonists such as McN-A-343 (27) and AF-102B (28) in the mouse hot-plate test. Since the doses of S-(-)-ET 126 and pirenzepine able to antagonise muscarinic antinociception are 100 times lower than the minimal dose of atropine

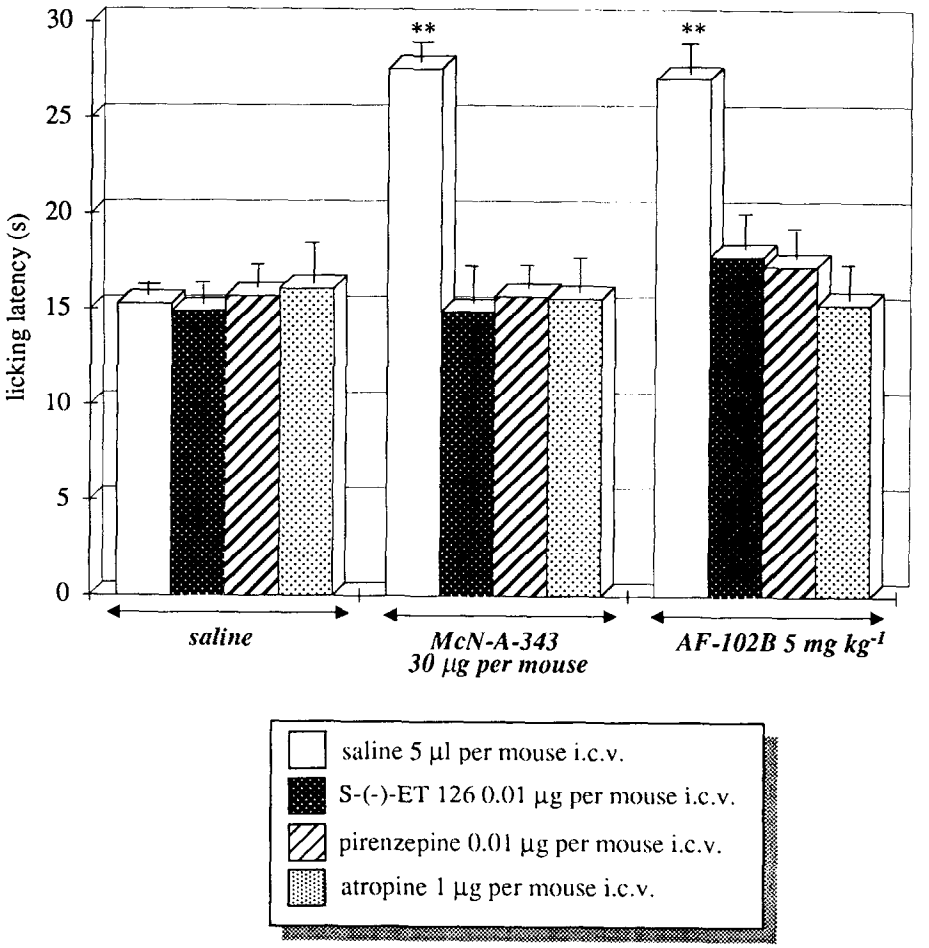

FIG. 5

Antagonism by S-(-)-ET-126, pirenzepine and atropine of McN-A-343 and AF-102B induced antinociception. The antagonistic effect was measured respectively 15 and 30 min after administration of analgesic drugs corresponding to the time of their maximum antinociceptive effect. Vertical lines represent s.e.m. The number of mice ranged between 8 and 20.
**P< 0.01 in comparison with saline treated animals.

necessary to prevent muscarinic antinociception (18) (Fig. 5), it is evident that the antagonism obtained at such low doses is selective for M_1 receptors.

In conclusion, we have shown that S-(-)-ET 126 is a potent and selective M_1 antagonist. This compound displays not only a higher affinity for muscarinic receptors but also a higher discriminative activity than pirenzepine and the recently reported M_1 antagonist spirotramine (29). Unlike pirenzepine and spirotramine, S-(-)-ET 126 is able to cross the blood brain barrier. The muscarinic antagonists available to date are not as capable of discriminating between

M_1/M_3 and quite often have difficulty in reaching the central nervous system. S-(-)-ET 126 is thus also active as an M_1 antagonist when injected in mice and represents useful tool for investigation into the physiological role of the M_1 muscarinic receptor subtype.

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