ANTINOCICEPTION INDUCED BY SM32 DEPENDS ON CENTRAL CHOLINERGIC MECHANISM

Pharmacological Research, Vol. 35, No. 2, 1997

ANTINOCICEPTION INDUCED BY SM 32 DEPENDS ON A CENTRAL CHOLINERGIC MECHANISM

CARLA GHELARDINI, NICOLETTA GALEOTTI, FULVIO GUALTIERI*, MARIA NOVELLA ROMANELLI* and ALESSANDRO BARTOLINI

*Department of Preclinical and Clinical Pharmacology, University of Florence, Viale G.B. Morgagni 65, I-50134 and *Department of Pharmaceutical Sciences, University of Florence, Via G. Capponi 9, I-50121 Florence, Italy*

Accepted 6 November 1996

The antinociceptive effect of SM 32 was examined in mice by using the hot-plate (10–40 mg kg^{-1} i.p; 3–30 μg per mouse i.c.v.) and abdominal constriction (10–30 mg kg^{-1} i.p) tests. In the antinociceptive dose-range, SM 32 did not impair mouse spontaneous motility and motor coordination evaluated respectively by the Animex and rota-rod tests. The increase in the pain threshold produced by SM 32 was prevented by dicyclomine, pirenzepine and hemicholinium-3 but not by naloxone and CGP 35348. *In vitro* experiments showed that the SM 32 amplified electrically- and nicotine-evoked guinea-pig ileum contractions. On the basis of the above data, it can be postulated that SM 32 exerts its antinociceptive effect through a potentiation of central cholinergic transmission.

KEY WORDS: antinociception, analgesia, ACh, cholinergic system.

INTRODUCTION

It has long been known that acetylcholine (ACh) [1], selective M_1 agonists such as McN-A-343 and AF-102B [2], unselective muscarinic agonists such as tremorine [3], oxotremorine [4, 5], arecoline [6], pilocarpine [7] and cholinesterase inhibitors such as physostigmine [8, 9] and diisopropyl fluorophosphate [10], induce antinociception in laboratory animals by activating the cholinergic system. Moreover, the amplification of cholinergic neurotransmission induced by antagonism of muscarinic autoreceptors [11, 12, 13] or, alternatively, by interaction with heteroreceptors [14] located on presynaptic cholinergic terminals, produces a central antinociceptive effect.

In order to obtain a new cholinergic amplifier, the compound labeled SM-32 ((±)-2-phenylthiobutyric acid α-tropyl ester oxalate) (Fig. 1) has been synthesized [15]. Romanelli *et al*. [16] demonstrated, using microdialysis technique, that SM 32 produced an increase in ACh release from the rat cerebral cortex *in vivo*. On the basis of the above reports, we decided to investigate the potential antinociceptive properties of SM 32.

Author for correspondence: Dr Carla Ghelardini, Department of Preclinical and Clinical Pharmacology, University of Florence, Viale G.B. Morgagni, 65, I-50134 Florence, Italy.

MATERIALS AND METHODS

Animals

Male Swiss albino mice (23–30 g) from Morini (San Polo d'Enza, Italy) and guinea-pigs (150–200 g) from Rodentia (Bergamo, Italy) breeding farms were used. Fifteen mice and four guinea-pigs were housed per cage. The cages were placed in the experimental room 24 h before the test for acclimatization. The animals were kept at 23±1°C with a 12 h light/dark cycle, light at 07:00 h, with food and water *ad libitum*. All experiments were carried out according to the guidelines of the European Community Council.

Hot plate test

The method adopted has been described by O'Callaghan and Holtzman [17]. Mice were placed inside a stainless steel container, thermostatically set at 52.5± 0.1°C in a precision water-bath from KW Mechanical Workshop, Siena, Italy. Reaction times (s), were measured with a stop-watch before and at regular intervals up to a maximum of 45 min after treatment. The endpoint used was the licking of the fore or hind paws. Mice with a licking latency below 12 and over 18 s in the pretest were rejected (30%). An arbitrary cut-off time of 45 s was adopted.

Abdominal constriction test

Mice were injected i.p. with a 0.6% solution of acetic acid (10 ml kg^{-1}), according to Koster *et al*. [18]. The number of stretching movements was

1043–6618/97/020141–07/$25.00/0/fr960110

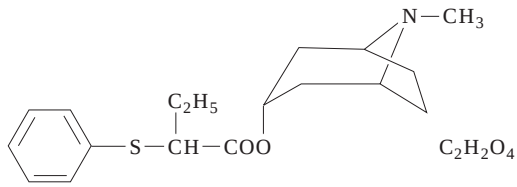

Fig. 1. Chemical structure of SM 32 ((±)-2-phenylthiobutyric acid α-tropyl ester oxalate).

counted for 10 min, starting 5 min after acetic acid injection.

Rota-rod test

The apparatus consisted of a base platform and a rotating rod of 3 cm diameter with a non-slippery surface. This rod was placed at a height of 15 cm from the base. The rod, 30 cm in length, was divided into five equal sections by six disks. Thus up to five mice were tested simultaneously on the apparatus, with a rod-rotating speed of 16 r.p.m. The integrity of motor coordination was assessed on the basis of endurance time of the animals on the rotating rod, expressed in seconds, according to Kuribara *et al*. [19]. One day before the test, the animals were trained twice. On the day of the test only the mice that were able to stay balanced on the rotating rod between 70 and 120 s (cut-off time) were selected for testing. The performance time was measured before and at various times after treatment.

Spontaneous activity meter (Animex)

Locomotor activity in mice was quantified using an Animex activity meter Type S (LKB, Farad, Sweden) set to maximum sensitivity. Every movement of mice, which were placed on the top of the Animex activity meter, produced a signal due to variation in inductance and capacity of the apparatus resonance circuit. Signals were then automatically converted to numbers. On the day of the experiment the mice were treated and then the cage, containing five mice, was put on the measuring platform. Activity counts were made every 15 min for 45 min starting immediately after injection of the drug. Because of the arbitrary scale adopted to quantify movements, drug-treated mice were always compared with saline-treated ones.

Isolated guinea-pig ileum

The myenteric plexus longitudinal muscle was prepared according to Paton and Vizi [20]. The strip was suspended in a 12.5 thermoregulated (36–37°C) bath and, after stabilization, the strip was stimulated electrically (0.1 Hz, 0.5 ms; double threshold voltage) or with nicotine (4 μM). The Krebs-Henseleit solution, bubbled with 95% O_2 and CO_2, had the following composition (mM): NaCl 118.0, KCl 4.7, $MgSO_4.7H_2O$ 1.2, $CaCl_2$ 2.5, KH_2PO_4 1.2, $NaHCO_3$ 25.0 and glucose 11.0.

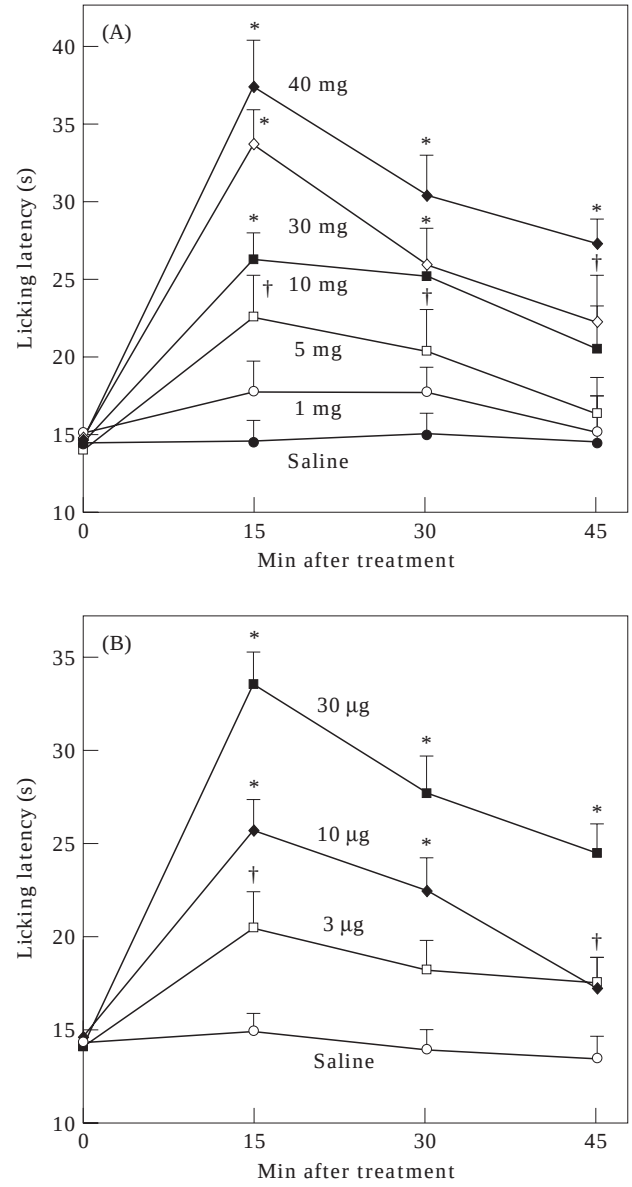

Fig. 2. Dose-response curves of SM 32 administered i.p. (A) and i.c.v (B) in the mouse hot-plate test. The doses are expressed as mg kg^{-1} i.p. and as μg per mouse i.c.v. Vertical lines show SEM †P<0.05; *P<0.01 in comparison with saline controls. Each point represents the mean of at least 10 mice.

Drugs

The following drugs were used: SM 32 was prepared according to Gualtieri *et al*. [15]; hemicholinium-3 hydrobromide (HC-3), pirenzepine dihydrochloride, naloxone hydrochloride, McN-A-343 (RBI); morphine hydrochloride (U.S.L. 10/D, Florence); dicyclomine dihydrochloride (Lepetit); baclofen, CGP 35348 (Ciba Geigy); oxotremorine (Fluka); nicotine hydrogentartrate (Sigma). Other chemicals were of the highest quality commercially available. All drugs were dissolved in isotonic (NaCl 0.9%) saline solution. Drug concentrations were prepared in such a way that the necessary dose could be administered in a volume of 10 ml kg^{-1} by intraperitoneal (i.p.) and subcutaneous (s.c.) route.

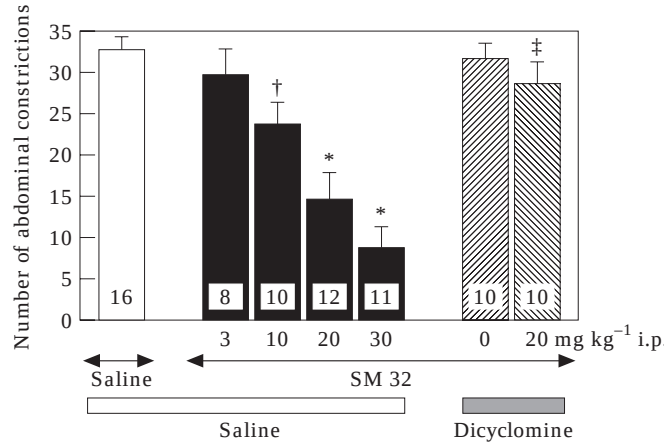

Fig. 3. Dose-response curves of SM 32 administered i.p. and antagonism exerted by dicyclomine (10 mg kg^{-1} i.p.) on antinociception induced by SM 32 (20 mg kg^{-1} i.p.) in the mouse abdominal constriction test induced by acetic 0.6% acid. The nociceptive responses were recorded 15 min after SM 32 administration. Dicyclomine was injected 30 min before testing. Vertical lines show SE mean. †P<0.05; *P<0.01 in comparison with saline controls. ‡P<0.01 in comparison with SM 32 (20 mg kg^{-1} s.c.). Numbers inside the columns indicate the number of mice.

Intracerebroventricular (i.c.v.) administration was performed under ether anaesthesia using isotonic saline as solvent, according to the method described by Haley and McCormick [21]. Briefly, during anaesthesia mice were grasped firmly by the loose skin behind the head. A hypodermic needle of 0.4 mm external diameter attached to a 10 μl syringe was inserted perpendicularly through the skull at a depth of no more than 2 mm into the brain of the mouse, where 5 μl were then administered. The injection site was 1.5 mm from either side of the midline on a line drawn through to the anterior base of the ears. To ascertain that the drugs were administered exactly into the cerebral ventricle, some mice were i.c.v. injected

Table I
Effects of pirenzepine, dicyclomine, hemicholinium-3 (HC-3), naloxone and CGP-35348 on antinociception induced by SM 32 (30 mg kg^{-1} i.p.) in the mouse hot-plate test

Pretreatment	*Treatment*	*No*	*Licking latency (s)*			
			Before pretreatment	*After treatment*		
				15 min	*30 min*	*45 min*
Saline 10 ml kg^{-1} i.p.	Saline	32	14.2±0.8	13.8±1.0	13.9±0.9	13.7±0.7
Saline 5 μl i.c.v.	Saline	16	14.1±0.9	15.2±1.1	14.3±0.8	14.3±0.5
Saline i.p. or i.c.v.	SM 32	43	13.6±0.8	32.5±1.2*	24.4±1.3*	22.1±1.5*
Pirenzepine	Saline	10	14.2±1.2	13.8±1.4	13.6±1.7	14.0±1.5
0.1 μg mouse i.c.v.	SM 32	10	15.0±0.7	17.9±2.2†	17.6±1.8†	16.5±1.3†
Dicyclomine	Saline	10	14.1±0.8	13.8±1.3	15.1±1.5	15.5±1.7
10 mg kg^{-1} i.p.	SM 32	10	13.8±0.9	16.7±2.1†	16.3±1.9†	16.2±2.1†
HC-3	Saline	14	14.2±0.5	14.3±1.2	14.8±1.2	15.3±1.6
1 μg mouse i.c.v.	SM 32	10	15.1±0.9	18.2±2.0†	17.7±1.7†	15.9±1.9†
Naloxone	Saline	11	14.9±0.7	13.3±1.4	14.5±1.2	14.7±1.3
3 mg kg^{-1} i.p.	SM 32	8	13.8±1.1	29.6±2.4*	25.2±1.7*	20.3±1.6‡
CGP 35348	Saline	10	14.0±0.8	12.7±1.6‡	13.4±1.7	13.9±1.2
100 kg^{-1} i.p.	SM 32	8	13.8±1.1	31.9±2.2*	25.6±2.1*	21.3±1.7*

*P<0.01; ‡P<0.05 in comparison with saline-saline;
†P<0.01 *vs* saline-SM 32 treated mice.

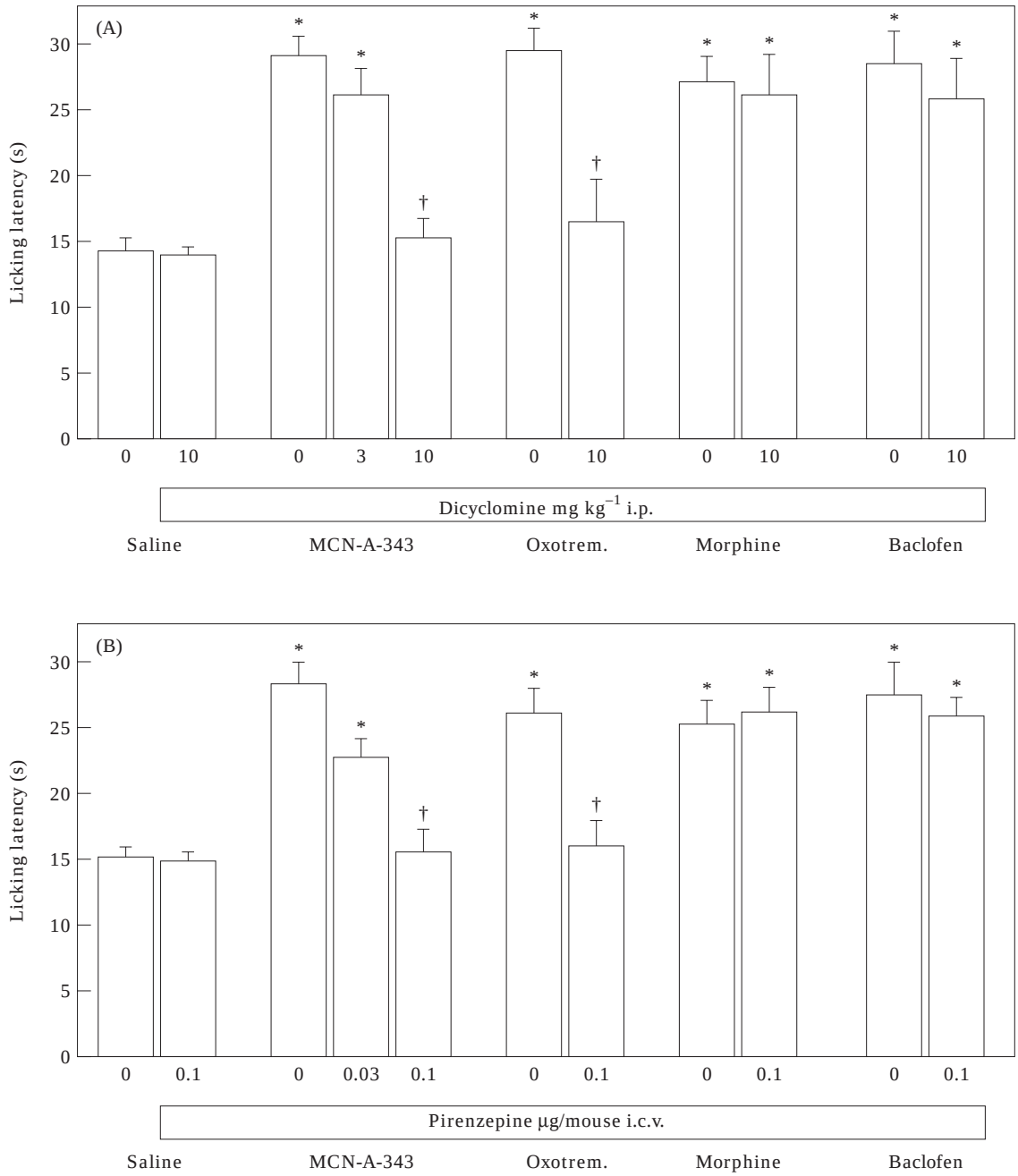

Fig. 4. Effect of dicyclomine (A) and pirenzepine (B) on antinociception induced by McN-A-343 (30 μg per mouse i.c.v.), oxotremorine (40 μg kg^{-1} s.c.), morphine (7 mg kg^{-1} s.c.) and baclofen (4 mg kg^{-1} s.c.) in the mouse hot-plate test. Dicyclomine and pirenzepine were injected 15 min before McN-A-343 and 1 min before oxotremorine, morphine and baclofen. Nociceptive responses were recorded 15 min after McN-A-343 injection and 30 min after oxotremorine morphine and baclofen injection. Each column represents the mean of at least 8 mice. Vertical lines show SEM. *P<0.01 in comparison with saline controls. †P<0.01 *vs* McN-A-343 or oxotremorine treated mice.

with 5 μl of diluted 1:10 Indian ink and their brains examined macroscopically after sectioning.

Statistical analysis

Results are given as the mean±SEM; analysis of variance (ANOVA), followed by Fisher's PLSD procedure for post-hoc comparison, was used to verify the significance between two means. P values of less than 0.05 were considered significant. Data were analysed with StatView for the Macintosh computer program (1992).

RESULTS

Antinociceptive activity of SM 32

SM 32, as shown in Fig. 2, produced a dose-dependent increase in the pain threshold in the mouse hot-

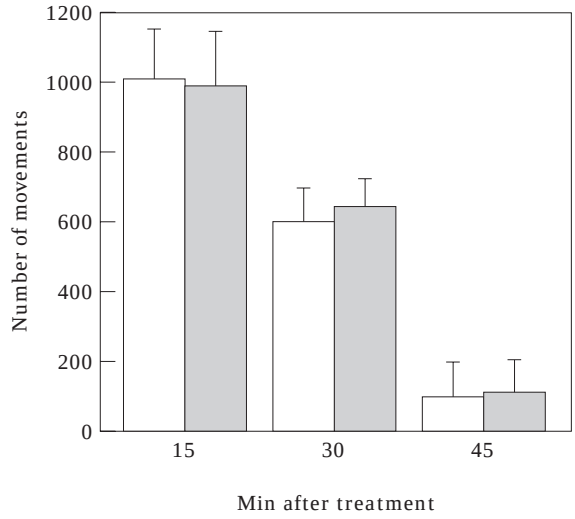

Fig. 5. Lack of effect by SM 32 on mouse spontaneous motility. Vertical lines show SEM. Each column represents the mean of 12 mice. □, Saline; ▨, SM 32 40 mg kg^{-1} i.p.

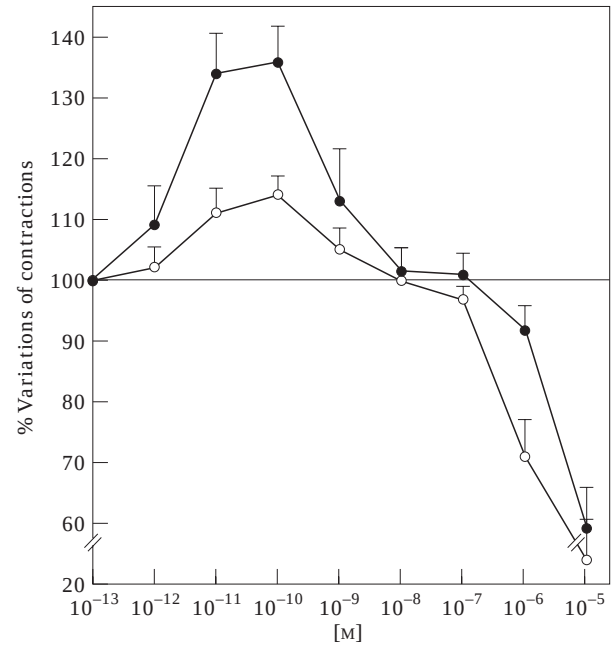

Fig. 6. SM 32 dose-response curves on nicotine (4×10^{-6} M) (●) and electrically (0.1 Hz; 0.5 ms; double threshold voltage) (○)-evoked contractions of guinea-pig ileum myenteric plexus longitudinal muscle strip expressed as percentage variation of contractions. Each point represents the mean of at least four experiments and vertical lines give SEM.

plate test after i.p. (10–40 mg kg^{-1}; panel A) and i.c.v. (3–30 μg per mouse; panel B) administration. The antinociceptive effect of SM 32 peaked 15 min after injection and then slowly diminished. Figure 3 illustrates the analgesic effect of SM 32 in the mouse acetic acid abdominal constriction test. SM 32 induced an increase in the pain threshold in a dose-dependent manner starting from the dose of 10 mg kg^{-1} i.p.

Antagonism of the SM 32 induced antinociception

In the mouse hot-plate test, the antinociceptive effect of SM 32 (30 mg kg^{-1} i.p.) was not antagonized by naloxone (3 mg kg^{-1} i.p.) and CGP-35348 (100 mg kg^{-1} i.p.) (Table I). Conversely, dicyclomine (10 mg kg^{-1} i.p.), pirenzepine (0.1 μg per mouse i.c.v.) and hemicholinium-3 (1 μg per mouse i.c.v.) were able to completely prevent SM 32 antinociception in the mouse hot-plate and abdominal constriction tests (Table I, Fig. 3). All antagonists were injected 15 min before SM 32, with the exception of CGP 35348, injected 5 min before SM 32.

Figure 4 shows that the doses of 10 mg kg^{-1} i.p. and 0.1 μg per mouse i.c.v. of dicyclomine and pirenzepine, respectively, were needed to completely antagonize the antinociception induced by the muscarinic agonists McN-A-343 and oxotremorine without interfering in any way with morphine (7 mg kg^{-1} s.c.) and baclofen (4 mg kg^{-1} s.c.) evoked analgesia.

Evaluation of the SM 32 effect on motor coordination and spontaneous motility

The motor coordination of mice treated with SM 32 was evaluated by using the rota rod test (Table II). The rota rod performance of mice treated with SM 32 at the dose of 40 mg kg^{-1} i.p. was not impaired in comparison with controls (Table II). On the contrary,

Table II
Effect of SM 32 in the rota-rod test

	Endurance time on rota-rod (s)			
	Before treatment	*After treatment*		
		15 min	*30 min*	*45 min*
Saline	99.5±6.3 (18)	93.6±6.1 (18)	101.8±4.6 (18)	106.5±5.4 (18)
SM 32 40 mg kg^{-1} i.p.	96.8±4.6 (10)	93.8±6.7 (10)	107.2±5.3 (10)	94.6±4.8 (10)
SM 32 60 mg kg^{-1} i.p.	105.4±4.9 (9)	65.8±7.2* (9)	55.8±7.5* (9)	74.3±8.2* (9)

*P<0.05 in comparison with saline controls. The number of mice is shown in parentheses.

SM 32 administered at the dose of 60 mg kg^{-1} i.p. significantly impaired rota-rod performance (Table II).

The spontaneous motility of mice was not modified by treatment with SM 32 (40 mg kg^{-1} i.p.) as revealed by the Animex apparatus (Fig. 5).

In vitro *functional studies*

As shown in Fig. 6 SM 32 added to the organ bath at concentrations ranging from 10^{-12}–10^{-9} M potentiated the contractions evoked by both nicotine (4 μM) and electrical stimulation. The effect was larger (area under the curve ratio) on the contractions induced by nicotine than that induced by electrical stimulation. The potentiation was no longer observed when the concentration of SM 32 in the medium was raised to 10^{-8} M. SM 32 began to inhibit both types of evoked contractions at 10^{-6} M.

DISCUSSION

SM 32 was able to induce antinociception in mice regardless of which noxious stimulus was used: thermal (hot-plate test) and chemical (abdominal constriction test). SM 32 antinociception was obtained without producing any visible modification in animal gross behavior. Moreover, motor coordination on the rota-rod test and normal spontaneous motility, as revealed by the Animex apparatus, were completely intact in mice treated with SM 32.

SM 32 exerted its antinociceptive effect by acting centrally. It was possible to enhance the pain threshold by injecting directly into the cerebral ventricles doses (30 μg per mouse) of SM 32 which were one thousand times lower than those needed parenterally.

SM 32 antinociception was found to be dependent on central cholinergic activation since it was prevented by the M_1-antagonists dicyclomine and pirenzepine and the ACh depletor HC-3. Taking into account that HC-3 and pirenzepine were able to antagonize SM 32 antinociception after i.c.v. injection, this supports the hypothesis that the analgesic site of action of SM 32 is localized in the central nervous system (CNS). A presynaptic mechanism facilitating cholinergic transmission is involved in SM 32 antinociception as revealed by the antagonism of HC-3. A postsynaptic mechanism of action can be ruled out since, as reported by Bartolini *et al*. [5, 2], HC-3 was not able to antagonize antinociception induced by agonists of postsynaptic muscarinic receptors such as oxotremorine, McN-A-343 and AF-102B.

The *in vitro* experiments supported the hypothesis that SM 32 amplifies cholinergic neurotransmission since SM 32, ranging from 10^{-12}–10^{-9}, increased presynaptically induced (electrical- and nicotine-evoked) contractions of longitudinal muscle of guinea-pig ileum. The amplification by SM 32 of the nicotine-evoked contractions of guinea-pig ileum, when compared to those elicited by electrical stimulation, depends on the inhibitory control exerted by noradrenaline, which is only released during electrical stimulation [13].

The hypothesis of a presynaptic cholinergic mechanism for SM 32 is in agreement with previous results demonstrating, by microdialysis studies, an increase in ACh release from the rat cerebral cortex induced by SM 32 administration [16].

Opioid and GABAergic neurotransmitter systems are not involved in SM 32 antinociception since the opioid antagonist naloxone and the $GABA_B$ antagonist CGP-35348 were unable to prevent the effect of SM 32. The doses and administration schedules of the above-mentioned drugs were ideal for preventing antinociception induced by morphine [14] and the $GABA_B$ agonist baclofen [22].

In conclusion, our results indicate that SM 32 is able to produce dose-dependent antinociception in mice, by potentiating endogenous cholinergic activity and without impairing motor coordination or spontaneous motility.

ACKNOWLEDGEMENTS

The authors wish to thank Ciba Geigy for the gift of CGP-35348. This research was supported by grants from Fidia S.p.A. (Aþano Terme, Italy) and from Ministero dell'Universita e della Ricerca Scientifica e Tecnologia (MURST).

REFERENCES

1. Pedigo NW, Dewey WL, Harris LS. Determination and characterization of the antinociceptive activity of intracerebroventricularly administered acetylcholine. *J Pharmacol Exp Ther* 1975; **193:** 845–52.
2. Bartolini A, Ghelardini C, Fantetti L, Malcangio M, Malmberg-Aiello P, Giotti A. Role of muscarinic receptor subtypes in central antinociception. *Br J Pharmacol* 1992; **105:** 77–82.
3. Lenke D. Narkosepotenzierende und analgestische Wirkung von 1,4-dipyrrolidino-2-butin. *Arch Exp Pathol Pharmacol* 1958; **234:** 35–45.
4. George R, Haslett WL, Jendel DJ. The central action of a metabolite of tremorine. *Life Sci* 1962; **1:** 361–3.
5. Bartolini A, Galli A, Ghelardini C, Giotti A, Malcangio M, Malmberg-Aiello P, Zucchi PL. Antinociception induced by systemic administration of local anaesthetics depends on a cholinergic mechanism. *Br J Pharmacol* 1987; **92:** 711–21.
6. Herz A. Wirkungen des Arecolins auf das Zentralnervensystem. *Arch Exp Pathol Pharmakol* 1962; **242:** 414–29.
7. Hendershot LC, Forsaith J. Antagonism of the frequency of phenylquinone-induced writhing in the mouse by weak analgesics and nonanalgesics. *J Pharmacol Exp Ther* 1959; **125:** 237–40.
8. Harris LS, Dewey WL, Howes J, Kennedy JS, Pars H. Narcotic antagonist analgesics: interaction with cholinergic systems. *J Pharmacol Exp Ther* 1969; **169:** 17–22.
9. Ireson C. A comparison of the antinociceptive action of

cholinomimetic and morphine-like drugs. *Br J Pharmacol* 1970; **40:** 92–101.
10. Lentz TL, Liley L, Michaelson U. Some actions of anticholinergic drugs. *Br J Pharmacol* 1969; **32:** 156–62.
11. Bartolini A, Ghelardini C, Gualtieri F, Malcangio M, Malmberg-Aiello P, Romanelli MN, Giotti A. I.c.v. AFDX 116 induces analgesia only when administered at very low doses. *Trends Pharmacol Sci* 1989; **Suppl. IV:** 99.
12. Gualtieri F, Ghelardini C, Giotti A, Malcangio M, Malmberg-Aiello P, Bartolini A. Analgesia induced by the M_2 antagonist methoctramine administered i.c.v. *Trends Pharmacol Sci* 1989; **Suppl. IV:** 99.
13. Ghelardini C, Malmberg-Aiello P, Giotti A, Malcangio M, Bartolini A. Investigation into atropine-induced antinociception. *Br J Pharmacol* 1990; **101:** 49–54.
14. Ghelardini C, Giotti A, Gualtieri F, Matucci R, Romanelli MN, Scapecchi S, Teodori E, Bartolini A. Presynaptic auto- and hetero-receptors in the cholinergic regulation of pain. In: Angeli P, Gulini U, Quaglia W, eds. *Trends in Receptor Research*. Amsterdam: Elsevier Science Publishers, 1992: pp. 95–114.
15. Gualtieri F, Bottalico C, Calandrella A, Dei S, Giovannoni P, Mealli S, Romanelli MN, Scapecchi S, Teodori E, Galeotti N, Ghelardini C, Bartolini A, Giotti A. Presynaptic cholinergic modulators as potent nootropic and analgesic drugs. II. 2-phenoxy, 2-phenylthio and 2-phenylamino alkanoic acid esters. *J Med Chem* 1994; **37:** 1712–9.
16. Romanelli MN, Bartolini A, Bertucci C, Dei S, Ghelardini C, Giovannini MG, Gualtieri F, Pepeu G, Scapecchi S, Teodori E. Synthesis, absolute configuration and cognition enhancing activity of SM-21, SM-32 and PG-9, new central ACh releasers. *Proceedings of II Int. Symposium 'Medicinal Chemistry Approaches to Alzheimer Disease'* 1995.
17. O'Callaghan JP, Holtzman SG. Quantification of the analgesic activity of narcotic antagonists by a modified hot-plate procedure. *J Pharmacol Exp Ther* 1975; **192:** 497–505.
18. Koster R, Anderson M, De Beer EJ. Acetic acid for analgesic screening. *Fed Proc* 1959; **18:** 412.
19. Kuribara H, Higuchi Y, Takadoro S. Effects of central depressants on rota-rod and traction performances in mice. *Jpn J Pharmacol* 1977; **27:** 117–26.
20. Paton WDM, Vizi ES. The inhibitory action of noradrenaline and acetylcholine output by guinea-pig longitudinal muscle strip. *Br J Pharmacol* 1969; **35:** 10–28.
21. Haley TJ, McCormick WG. Pharmacological effects produced by intracerebral injection of drugs in the conscious mouse. *Br J Pharmacol Chemother* 1957; **12:** 12–5.
22. Malcangio M, Ghelardini C, Giotti A, Malmberg-Aiello P, Bartolini A. CGP 35348, a new $GABA_B$ antagonist, prevents antinociception and muscle-relaxant effect induced by baclofen. *Br J Pharmacol* 1991; **103:** 1303–8.