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Antinociceptive and Antiamnesic Properties of the Presynaptic Cholinergic Amplifier PG-9[1]

CARLA GHELARDINI, NICOLETTA GALEOTTI, FULVIO GUALTIERI, VITTORIO MARCHESE, CRISTINA BELLUCCI and ALESSANDRO BARTOLINI

Department of Pharmacology (C.G., N.G., V.M., A.B.), University of Florence, Viale G.B. Morgagni 65, I-50134 and Department of Pharmaceutical Sciences (F.G., C. B.), University of Florence, Via G. Capponi 9, I-50121 Florence, Italy

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ABSTRACT

The antinociceptive effect of 3α-tropyl 2-(*p*-bromophenyl)propionate [(±)-PG-9] (10–40 mg kg^{-1} s.c.; 30–60 mg kg^{-1} p.o.; 10–30 mg kg^{-1} i.v.; 10–30 μg/mouse i.c.v.) was examined in mice, rats and guinea pigs by use of the hot-plate, abdominal-constriction, tail-flick and paw-pressure tests. (±)-PG-9 antinociception peaked 15 min after injection and then slowly diminished. The antinociception produced by (±)-PG-9 was prevented by the unselective muscarinic antagonist atropine, the M_1-selective antagonists pirenzepine and dicyclomine and the acetylcholine depletor hemicholinium-3, but not by the opioid antagonist naloxone, the γ-aminobutyric acid$_B$ antagonist 3-aminopropyl-diethoxy-methyl-phosphinic acid, the H_3 agonist *R*-(α)-methylhistamine, the D_2 antagonist quinpirole, the 5-hydroxytryptamine$_4$ antagonist 2-methoxy-4-amino-5-chlorobenzoic acid 2-(diethylamino)ethyl ester hydrochloride, the 5-hydroxytryptamine$_{1A}$ antagonist 1-(2-methoxyphenyl)-4-[4-(2-phthalimido)butyl]piperazine hydrobromide and the polyamines depletor reserpine. Based on these data, it can be postulated that (±)-PG-9 exerted an antinociceptive effect mediated by a central potentiation of cholinergic transmission. (±)-PG-9 (10–40 mg kg^{-1} i.p.) was able to prevent amnesia induced by scopolamine (1 mg kg^{-1} i.p.) and dicyclomine (2 mg kg^{-1} i.p.) in the mouse passive-avoidance test. Affinity profiles of (±)-PG-9 for muscarinic receptor subtypes, determined by functional studies (rabbit vas deferens for M_1, guinea pig atrium for M_2, guinea pig ileum for M_3 and immature guinea pig uterus for putative M_4), have shown an M_4/M_1 selectivity ratio of 10.2 that might be responsible for the antinociception and the antiamnesic effect induced by (±)-PG-9 through an increase in acetylcholine extracellular levels. In the antinociceptive and antiamnesic dose range, (±)-PG-9 did not impair mouse performance evaluated by the rota-rod test and Animex apparatus.

The activation of the cholinergic system induces antinociception in laboratory animals (Pedigo *et al.*, 1975; George *et al.*, 1962; Herz, 1962; Hendershot and Forsaith, 1959; Harris *et al.*, 1969) and humans (Hood *et al.*, 1995). Bartolini *et al.* (1992) 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, whereas the M_2-selective agonist arecaidine propargil ester (APE) was not. Moreover, Bartolini *et al.* (1992) 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. It has also been reported that the antimuscarinic drug atropine, at very low doses, produces a cholinomimetic effect by inducing a central cholinergic antinociception in laboratory animals regardless of the route of administration and the noxious stimulus applied (Ghelardini *et al.*, 1990). This paradoxical effect of atropine confirmes the previous observations made by Ferguson-Anderson (1952) that reported that the tincture of belladonna in small doses, given by mouth, had a parasympathomimetic action increasing the frequency and amplitude of gastric contractions.

The typical cholinergic symptomatology (tremors, sialorrhea, diarrhea, lacrimation, etc.) did not accompany the antinociceptive activity of atropine. The atropine-induced increase in the pain threshold was attributable to the *R*-(+)-enantiomer of atropine, *R*-(+)-hyoscyamine, because *S*-(−)-

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ABBREVIATIONS: i.c.v., intracerebroventricular; s.c., subcutaneous; i.p., intraperitoneal; p.o., per os; i.v., endovenous; PG-9, 3α-tropyl 2-(*p*-bromophenyl)propionate; McN-A-343, 4-(N-[3-chlorophenyl]-carbamoyloxy)-2-butynyl-trimethylammonium chloride; AFDX-116, 11,2-(diethylamino)methyl-1-piperidinil acetyl-5,11-dihydro-6H-pyrido 2,3-b 1,4 benzodiazepine-6-one; CGP 35348, 3-aminopropyl-diethoxy-methyl-phosphinic acid; RAMH, (*R*)-α-methylhistamine; NAN-190, (1-(2-methoxyphenyl)-4-[4-(2-phthalimido)butyl]piperazine hydrobromide); SDZ 205557, (2-methoxy-4-amino-5-chlorobenzoic acid 2-(diethylamino) ethyl ester hydrochloride); 5-HT, 5-hydroxytryptamine; ACh, acetylcholine; HC-3, hemicholinium-3 hydrobromide; GABA, γ-aminobutyric acid.

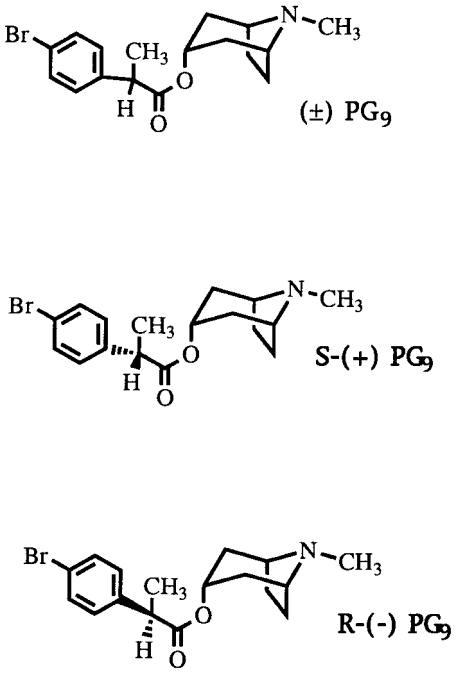

Fig. 1. Chemical structure of PG-9 racemate and enantiomers.

hyoscyamine was ineffective in all antinociceptive tests used (Ghelardini *et al.*, 1992). The investigation of the antinociceptive effect of atropine demonstrated, by microdialysis techniques, that *R*-(+)-hyoscyamine, at effective doses, produced an increase in the ACh release from the rat cerebral cortex *in vivo* (Ghelardini *et al.*, 1997). On the basis of the above-mentioned results, the racemate (Gualtieri *et al.*, 1994) and the enantiomers (Romanelli *et al.*, 1995) of the compound, PG-9, structurally related to atropine (fig. 1), have been synthesized to obtain a new cholinergic amplifier endowed with more intensive antinociceptive activity than atropine but as lacking in cholinergic side effects as atropine. For this purpose, (±)-PG-9 antinociceptive properties were investigated by use of the hot-plate, abdominal-constriction, paw-pressure and tail-flick tests, whereas the incidence of behavioral side effects was detected by the rota-rod test and Animex apparatus. Furthermore, the central cholinergic system has long been known to be involved in the modulation of learning and memory processes in animals and man. Drugs that affect the central cholinergic system have been found either to enhance or to hinder performance in learning and memory tests. Direct muscarinic agonists (oxotremorine, arecoline, AF-102B, RS 86, etc.), acetylcholine esterase inhibitors (physostigmine, diisopropyl fluorophosphate,

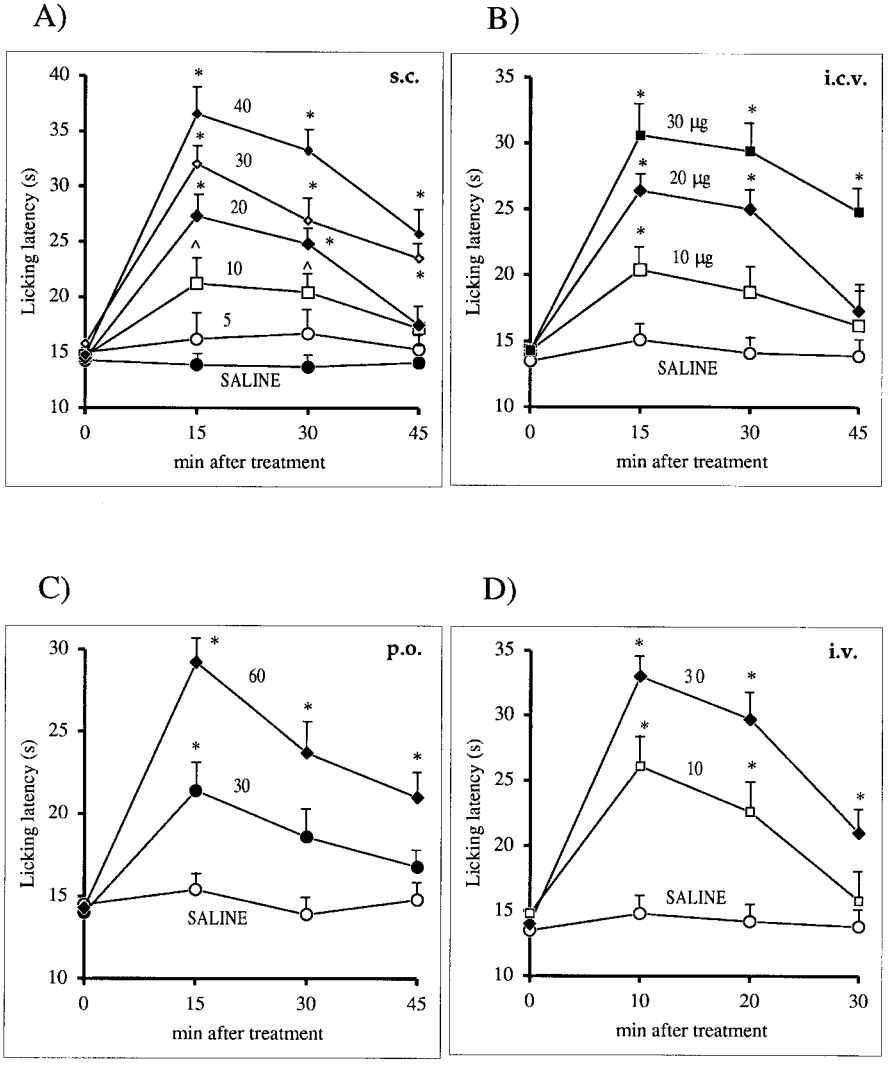

Fig. 2. Dose-response curves of (±)-PG-9 administered s.c. (A), i.c.v. (B), p.o. (C) and i.v. (D) in the mouse hot-plate test. The doses are expressed as milligrams per kilogram s.c., p.o. and i.v. and as micrograms per mouse i.c.v. Vertical lines show S.E.M. $P < .05$; *$P < .01$ in comparison with saline controls. Each point represents the mean of at least 10 mice.

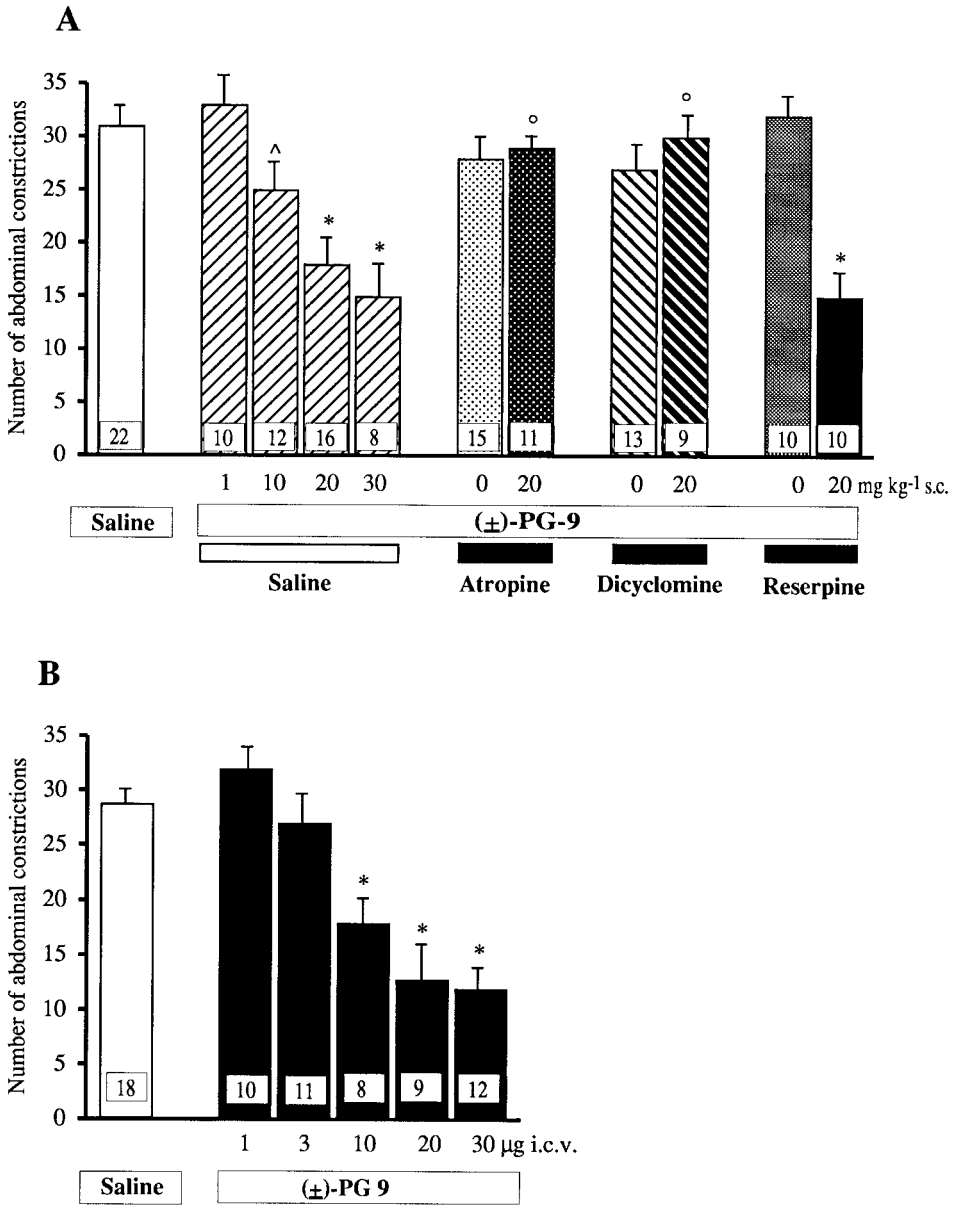

Fig. 3. (A) Dose-response curve of (±)-PG-9 administered s.c. and effect of atropine (5 mg kg^{-1} i.p.), dicyclomine (10 mg kg^{-1} i.p.) and reserpine (2 mg kg^{-1} i.p.) pretreatments on antinociception induced by (±)-PG-9 (20 mg kg^{-1} s.c.) in the mouse abdominal constriction test induced by 0.6% acetic acid. Atropine, dicyclomine and reserpine were injected, respectively, 15 min, 15 min and twice 48 and 24 h before (±)-PG-9 administration. The nociceptive responses were recorded 15 min after (±)-PG-9 administration. (b) Dose-response curve of (±)-PG-9 administered i.c.v. Vertical lines show S.E.M $P < .05$; *$P < .01$ in comparison with saline controls. °$P < .01$ in comparison with (±)-PG-9 (20 mg kg^{-1} s.c.). Numbers inside the columns indicate the number of mice.

eptastigmine, tacrine, etc.) and acetylcholine releasers (AFDX 116, DuP 996, etc.) potentiate test performance retention in rodents (Coyle, 1995). On the contrary, disruption of the cholinergic system impairs cognitive processes. The administration of muscarinic antagonists (scopolamine, atropine, pirenzepine and dicyclomine), inhibitors of choline uptake (hemicolinium-3) or lesions of nucleus basalis magnocellularis or injection of the cholinotoxic agent AF64A, all induce amnesia (Coyle, 1995). Considering that *R*-(+)-hyoscyamine was able to prevent amnesia induced by both scopolamine and dicyclomine in mice (Ghelardini *et al.*, 1997), the potential antiamnesic activity of (±)-PG-9 was investigated with the mouse passive-avoidance test.

Methods

Animals

Male Swiss albino mice (23–30 g) and Wistar rats (200–300 g) from Morini (San Polo d'Enza, Italy), Fisher 344 rats (200–300 g) from Charles River (Calco, Italy) and guinea pigs (150–200 g) from Rodentia (Bergamo, Italy) breeding farms were used. Fifteen mice and four rats or 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 7 A.M., with food and water *ad libitum*. All experiments were carried out according to the guidelines of the European Community Council.

Analgesic Tests

Hot-plate test. The method adopted was described by O'Callaghan and Holzman (1975). 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. Those mice scoring less than 12 and more than 18 s in the pretest were rejected (30%). An arbitrary cut-off time of 45 s was adopted.

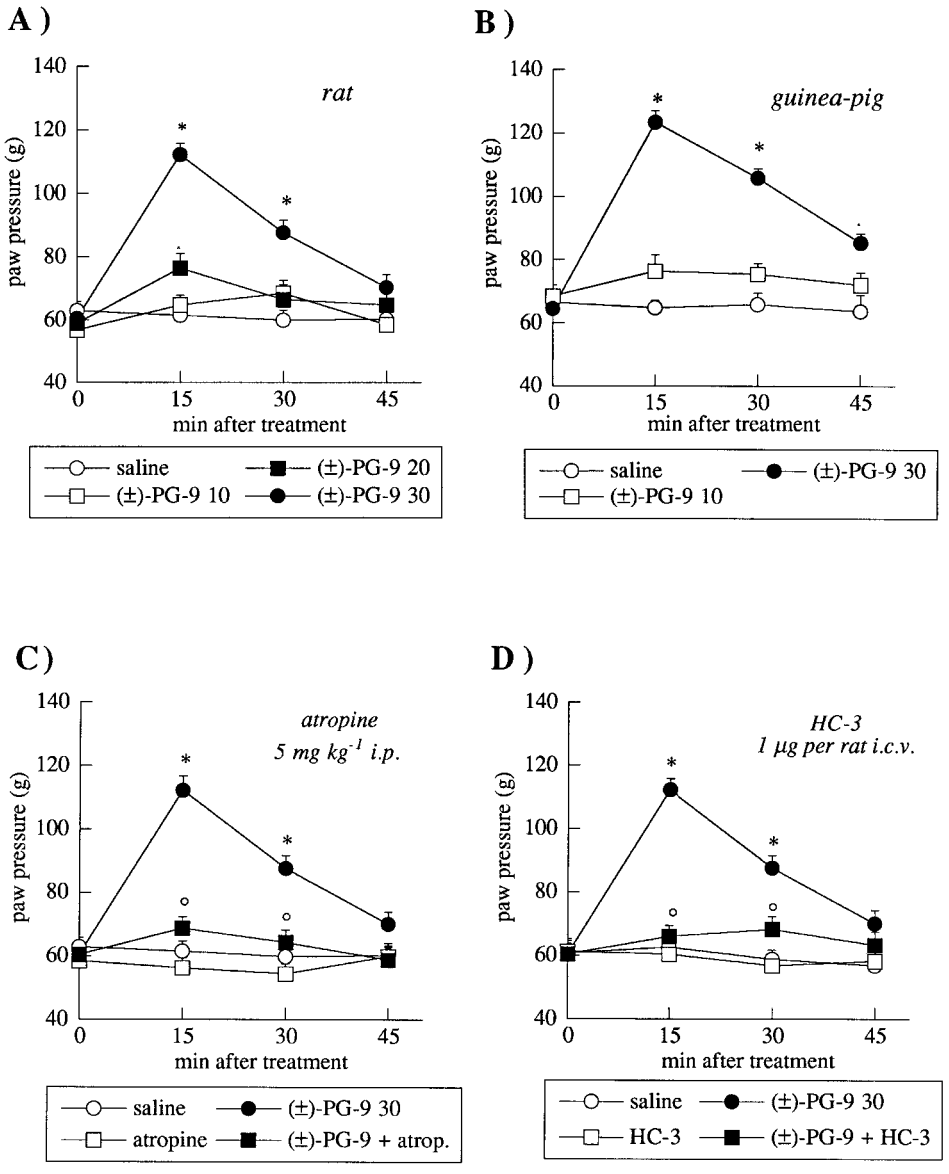

Fig. 4. Dose-response curve of i.p. (±)-PG-9 in the rat (A) and guinea-pig (B) paw pressure test and antagonism exerted by atropine (5 mg kg^{-1} i.p.; C) and HC-3 (1 µg/mouse i.c.v.; D) in the rat paw pressure test. Atropine and HC-3 were injected respectively 15 min and 5 h before (±)-PG-9 administration. Vertical lines show S.E.M. *P < .01 in comparison with saline controls; °P < .01 in comparison with (±)-PG-9 (30 mg kg^{-1} i.p.). Each point represents the mean of at least 5 rats and 4 guinea pigs. The doses of (±)-PG-9 are expressed as milligrams per kilogram i.p.

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.* (1959). The number of stretching movements was counted for 10 min, starting 5 min after acetic acid injection.

Paw-pressure test. The nociceptive threshold in the rat and guinea pig was determined with an analgesimeter (Ugo Basile, Varese, Italy), according to the method described by Leighton *et al.* (1988). Threshold pressure was measured before treatment and 15, 30 and 45 min after treatment. Rats and guinea pigs scoring less than 30 g or more than 85 g during the test and before drug administration were rejected (25%). An arbitrary cut-off value of 250 g was adopted.

Tail-flick test. An analgesimeter from Ugo Basile (Varese, Italy) was used to perform the tail- flick test described by D'Amour and Smith (1941). The light from a project bulb, situated beneath the platform where the rat was placed, was focused through a small hole on the ventral part of the tail at a point about 4 cm from the tip. Withdrawal of the tail exposed a photocell to the light, which turned off the thermal stimulus and automatically stopped the clock. The intensity was regulated so that the reaction time varied between 2 and 4 s. The analgesia was tested before and 15, 30 and 45 min after treatment of rats. Each value was derived from the mean of three consecutive readings in which the light was focused on three adjacent points of the tail. An arbitrary cut-off value of 10 s was adopted.

Antiamnesic Test: Passive-Avoidance Test

The test was performed according to the step-through method described by Jarvik and Kopp (1967), as we modified it for testing drugs endowed with analgesic properties. The apparatus consists of a two-compartment acrylic box with a lighted compartment connected to a darkened one by a guillotine door. In the original method mice received a punishing electrical shock as soon as they entered the dark compartment, whereas in our modified method, after entry into the dark compartment, mice receive a nonpainful punishment consisting of a fall into a cold water bath (10°C). For this purpose the dark chamber was constructed with a pitfall floor. The latency times for entering the dark compartment were measured in the training test and after 24 h in the retention test. For memory disruption, mice were injected i.p. with the amnesic drugs scopolamine and dicyclomine. (±)-PG-9, physostigmine and piracetam were injected 20 min before the training session, whereas scopolamine and dicyclomine were injected immediately after termination of the training session. The maximum entry latency allowed in the retention session was

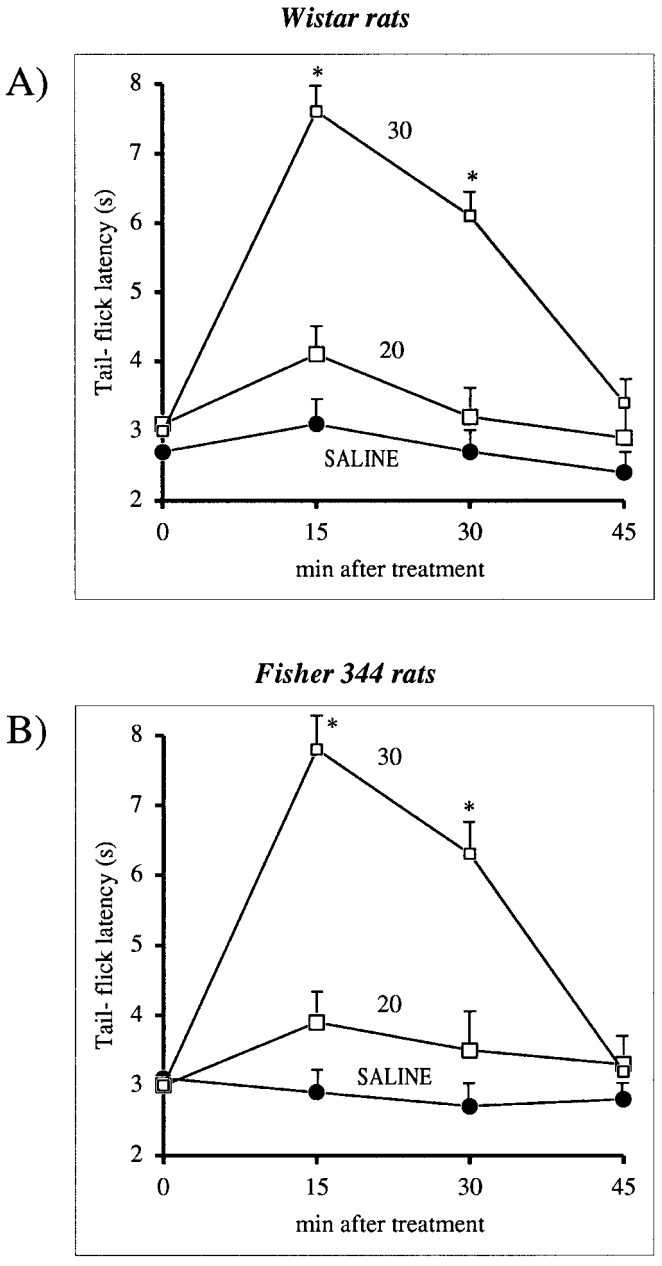

Fig. 5. Dose-response curves of (±)-PG-9 administered i.p. in the Wistar rats (A) and in the Fisher 344 rats (B) in the tail-flick test. The doses are expressed as milligrams per kilogram i.p. Vertical lines show S.E.M. *P < .01 in comparison with saline controls. Each point represents the mean of at least six rats.

120 s. The memory degree of received punishment (fall into cold water) was expressed as the increase in seconds between training and retention latencies.

Additional Behavioral Tests

Spontaneous activity meter (Animex). Locomotor activity in mice was quantified with an Animex activity meter Type S (LKB, Farad, Sweden) set to maximum sensitivity. Mice were placed on the top of the Animex activity meter and each movement produced a signal caused by variation in inductance and capacity of the apparatus resonance circuit. These signals were 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 measurement 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.

Rota-rod test. The apparatus consisted of a base platform and a rotating rod of 3-cm diameter with a nonslippery surface. The 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 based on the number of falls from the rod in 30 s according to Vaught *et al.* (1985). The performance time was measured before and 15, 30 and 45 min after treatment.

In Vitro Functional Studies

Isolated rabbit vas deferens (M_1). Experiments on isolated rabbit vas deferens were performed according to the method described by Eltze (1988) and modified by Dei *et al.* (1995). The preparations were maintained at 32°C and tissues were stimulated through platinum electrodes by square-wave pulses (2 ms, 0.1 Hz, 10–30 V). Contractions were measured isometrically after tissues had been equilibrated for 1 h, then a cumulative dose-response curve for the inhibitory effect of McN-A-343 was plotted.

Isolated guinea-pig left atria (M_2). Isolated left atria were prepared according to the method described by Eltze *et al.* (1985) and modified by Dei *et al.* (1995). Bath fluid temperature was maintained at 30°C. Atria were stimulated electrically (1 Hz, 1 ms, 4–10 V) by means of two platinum electrodes. Carbachol negative inotropic effects on isometric atria contractions were recorded before and 1 h after perfusion with antagonists.

Isolated guinea pig ileum (M_3). Isolated ileum fragments were prepared according to Eltze and Figala (1988). Bath fluid temperature was maintained at 37°C. Isotonic ileum contractions induced by ACh were recorded before and 1 h after perfusion with antagonists.

Guinea pig isolated uterus (M_4). Experiments on isolated immature guinea pig uterus were performed according to Dörje *et al.* (1990). The preparations were maintained at 30°C and after 1 h equilibration period isotonic contractions to carbachol were recorded. Initially the tissues were exposed to a single concentration of carbachol (3 nmol l^{-1}) to check the responsiveness to the agonist, and a dose-response curve for carbachol was obtained.

Determination of antagonist affinities. After a stabilization for 30 to 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 during the period required for the determination of two concentration-response curves. 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 absence and presence of antagonists were determined graphically for the calculation of dose ratios.

Acetylcholinesterase activity. Acetylcholinesterase activity was assayed according to Ellman *et al.* (1961), with 0.5 mM acetylthiocholine iodide as substrate. The (±)-PG-9 inhibitory effect was tested at various concentrations on a purified preparation of acetylcholinesterase from the electric eel.

Drugs

The following drugs were used: PG-9 racemate was prepared according to Gualtieri *et al.* (1994); *R*-(+)-PG-9 and *S*-(−)-PG-9 were prepared according to Romanelli *et al.* (1995); *R*-(+)-hyoscyamine was prepared according to Gualtieri *et al.* (1991); SDZ 205557 was prepared in the Department of Pharmaceutical Sciences of the University of Florence, Italy, according to the method described by Romanelli *et al.* (1993); atropine sulfate, carbamylcholine chloride, physostigmine hemisulfate and yohimbine hydrochloride (Sigma, Milan, Italy), HC-3, pirenzepine dihydrochloride, naloxone hydrochloride, quinpirole hydrochloride, (*R*)-α-methylhistamine dihydro-

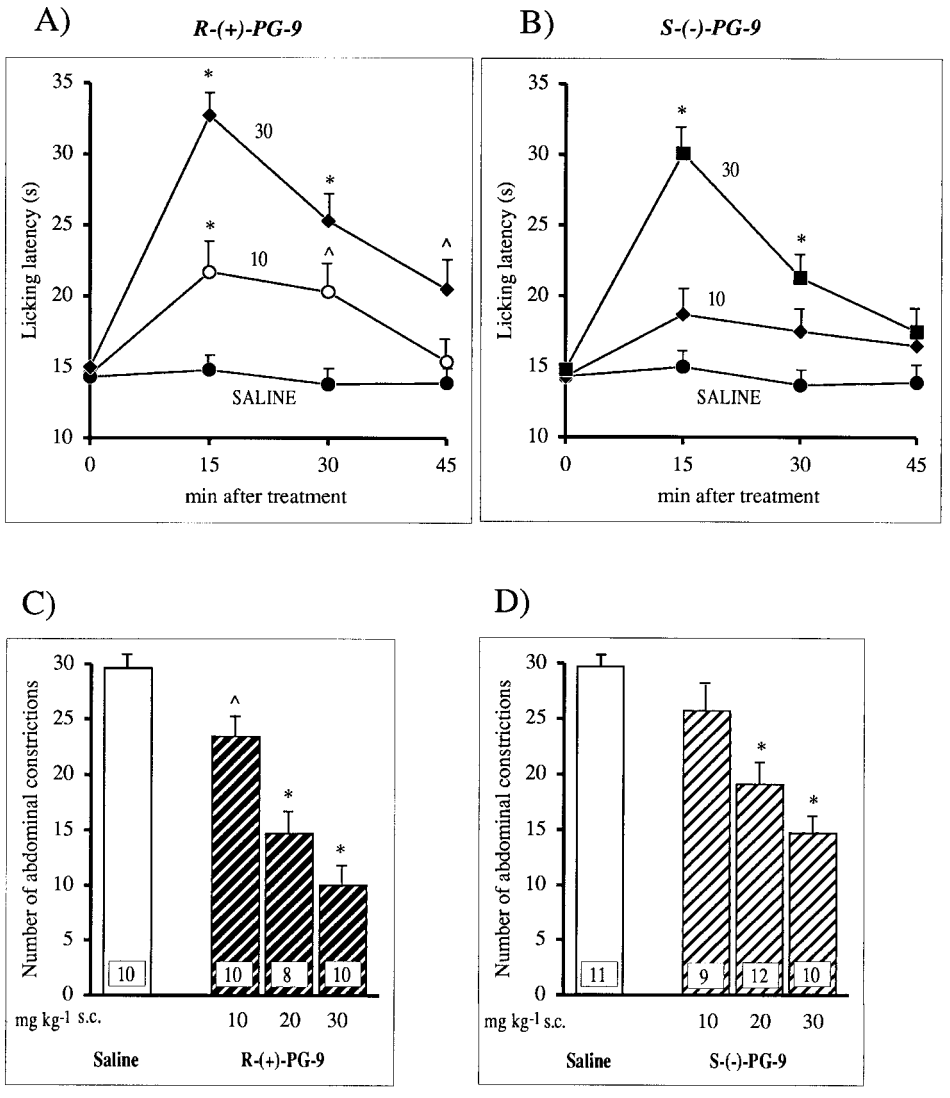

Fig. 6. Dose-response curves of *R*-(+)-PG-9 (A, C) and *S*-(−)-PG-9 (B, D) in the mouse hot-plate test and abdominal-constriction test. The doses are expressed as milligrams per kilogram s.c. Vertical lines show S.E.M. $P < .05$; *$P < .01$ in comparison with saline controls. The number of mice is shown in columns.

chloride, NAN 190, McN-A-343 (R.B.I., Milan, Italy); acetylcholine chloride (Merck, Rome, Italy); morphine hydrochloride (U.S.L. 10/D, Florence, Italy), diphenhydramine hydrochloride and AFDX-116 (De Angeli, Milan, Italy); clomipramine hydrochloride (anafranil), CGP 35348 and reserpine (Ciba Geigy, Basel, Switzerland); dicyclomine hydrochloride (Le Petit, Milan, Italy). Other chemicals were of the highest quality commercially available. All drugs were dissolved in isotonic (0.9% NaCl) saline solution or dispersed in 1% sodium carboxymethylcellulose immediately before use, except reserpine which was dissolved in a 20% solution of ascorbic acid and *R*-(+)-hyoscyamine that was dissolved in 0.1 M HCl and then diluted with saline (1:10). Drug concentrations were prepared so that the necessary dose could be administered in a volume of 10 ml kg^{-1} by s.c., i.p. and p.o. routes or 5 ml kg^{-1} by the i.v. route. Intracerebroventricular administration was performed under ether anesthesia with isotonic saline as solvent, according to the method described by Haley and McCormick (1957) for mice and which we adapted for rats. During anesthesia, mice and rats 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 at a depth of no more than 2 mm into the brain of the mouse and 4 mm into the brain of the rat, where 5 μl (mice) or 10 μl (rats) were then administered. The injection site was 1.5 mm (mice) or 2.5 mm (rats) from either side of the middle of 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 and rats were injected i.c.v. with 5 to 10 μl of diluted 1:10 Indian ink and their brains examined macroscopically after sectioning. The accuracy of the injection technique in both mice and rats was evaluated, and 95% of the injections were correct.

Statistical Analysis

Results are given as the mean ± S.E.M.; analysis of variance, followed by Fisher's Protected Least Significant Difference procedure for *post hoc* comparison, was used to verify the significance between two means. P values of less than .05 were considered significant. Data were analyzed with the StatView for the Macintosh computer program (1992).

Results

Antinociceptive activity of PG-9. (±)-PG-9, as shown in figure 2, produced a dose-dependent increase in the pain threshold in the mouse hot-plate test after s.c. (10–40 mg kg^{-1}; fig. 2A), i.c.v. (10–30 μg/mouse; fig. 2B), p.o. (30–60 mg kg^{-1}; fig. 2C) and i.v. (10–30 mg kg^{-1}; fig. 2D) administration. The antinociceptive effect of (±)-PG-9, regardless of the route of administration, peaked 15 min after injection and then slowly diminished. Figure 3, A and B, illustrates the

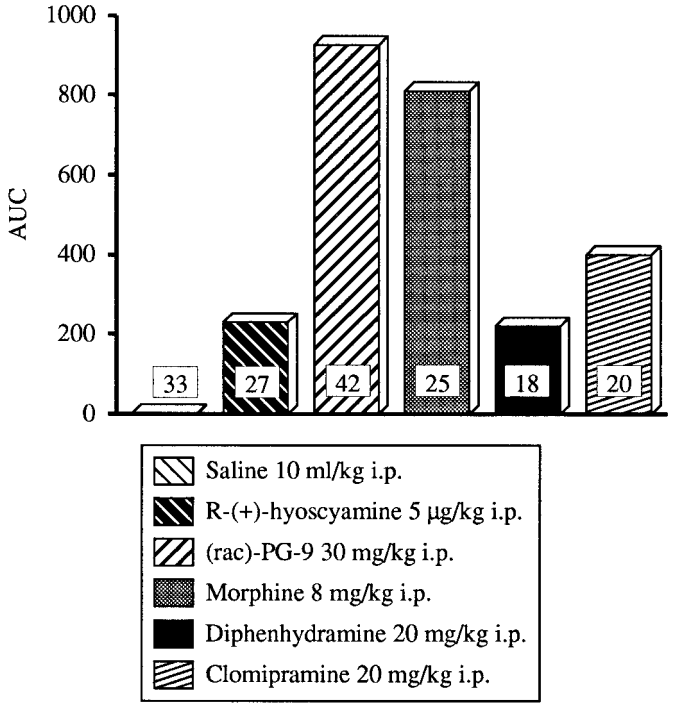

Fig. 7. Area under the curve (AUC) of the analgesic effect of (±)-PG-9 in comparison with *R*-(+)-hyoscyamine, morphine, diphenhydramine and clomipramine in the mouse hot-plate test. AUC represents the sum of four measurements. Antinociceptive responses were evaluated every 15 min and exactly 15, 30, 45 and 60 min after administration for *R*-(+)-hyoscyamine, diphenhydramine and clomipramine and 30, 45, 60 and 75 min after morphine injection. Numbers inside the columns indicate the number of mice.

analgesic effect of (±)-PG-9 in the mouse acetic acid abdominal constriction test. (±)-PG-9 induced an increase in the pain threshold in a dose-dependent manner starting from the dose of 10 mg kg^{-1} s.c. (fig. 3A). (±)-PG-9 showed antinociceptive properties also after the injection of 10 to 30 μg/mouse i.c.v. (fig. 3B).

(±)-PG-9 was able to increase the pain threshold not only in mice but also in rats and guinea pigs. In the paw-pressure test (±)-PG-9, administered i.p. at the dose of 20 to 30 mg kg^{-1} in the rat (fig. 4A) and 30 mg kg^{-1} in guinea pigs (fig. 4B), reached maximum antinociception 15 min after injection and then slowly diminished. The analgesic profile of (±)-PG-9 was also investigated in Wistar and Fisher 344 rat strains by using the tail-flick test (fig. 5). In both rat strains used (±)-PG-9 exhibited similar antinociceptive activity, peaking 15 min after i.p. injection of 30 mg kg^{-1} (fig. 5).

The analgesic effect of the two enantiomers of *R*-(+)-PG-9 and *S*-(−)-PG-9 was evaluated in the mouse hot-plate test (fig. 6, A and B) and in the acetic acid abdominal-constriction test (fig. 6, C and D). Both enantiomers dose-dependently increased the pain threshold, although *R*-(+)-PG-9 was slightly more effective than *S*-(−)-PG-9.

The areas under the curve of the antinociception induced by (±)-PG-9 (30 mg kg^{-1} i.p.), *R*-(+)-hyoscyamine (5 μg kg^{-1} i.p.), morphine (8 mg kg^{-1} i.p.), diphenhydramine (20 mg kg^{-1} i.p.) and clomipramine (25 mg kg^{-1} i.p.) are reported in figure 7. The doses of the analgesic drugs chosen were the highest that did not impair rota-rod performance.

Antagonism of the (±)-PG-9 induced antinociception. In the mouse hot-plate test, the antinociceptive effect of (±)-PG-9 (30 mg kg^{-1} s.c.) was not antagonized by naloxone (1 mg kg^{-1} i.p.; fig. 8D), CGP-35348 (2.5 μg/mouse i.c.v.), (*R*)-α-methylhistamine (10 mg kg^{-1} i.p.), quinpirole (0.1 mg kg^{-1} i.p.), SDZ-205557 (10 mg kg^{-1} i.p.), NAN 190 (0.5 μg/mouse i.c.v.) (data not shown) and, in the abdominal-constriction test, by reserpine (2 mg kg^{-1} i.p.) (fig. 3). Conversely, atropine (5 mg kg^{-1} i.p.), pirenzepine (0.1 μg/mouse i.c.v.) and hemicolinium-3 (1 μg/mouse or rat i.c.v.) were able to completely prevent (±)-PG-9 antinociception in the mouse hot-plate (fig. 8, A–C), abdominal-constriction (fig. 3A) and rat paw-pressure tests (fig. 4, C and D). All antagonists were injected 15 min before (±)-PG-9, with the exception of reserpine (injected twice 48 and 24 h before the test), HC-3 (injected 5 h before the test) and CGP 35348 (injected 5 min before (±)-PG-9).

Antiamnesic activity of (±)-PG-9. Pretreatment with (±)-PG-9 (10–30 mg kg^{-1} i.p.), injected 20 min before the training session, prevented the amnesia induced by scopolamine (1 mg kg^{-1} i.p.) and dicyclomine (2 mg kg^{-1} i.p.) in the mouse passive-avoidance test. (±)-PG-9 enhanced the entrance latency up to a value similar to that produced by control animals (fig. 9). (±)-PG-9, at 1 mg kg^{-1} i.p., was completely ineffective (fig. 9). The antiamnesic effect of (±)-PG-9 was equal to that produced by the cholinesterase inhibitor physostigmine (0.2 mg kg^{-1} i.p.) and the nootropic drug piracetam (30 mg kg^{-1} i.p.).

(±)-PG-9, when given alone, at the highest doses used, had no effect on the mouse passive-avoidance test in comparison with saline-treated mice (fig. 9), nor were there any differences in the entrance latencies for each group in the training session of the passive- avoidance test (data not shown).

Evaluation of the PG-9 effect on spontaneous activity and motor coordination. The motor coordination of mice treated with (±)-PG-9, *R*-(+)-PG-9 and *S*-(-)-PG-9 was evaluated by use of the rota-rod test (table 1), whereas their spontaneous activity was investigated by use of the Animex apparatus. The rota-rod performance of mice treated with (±)-PG-9 at the dose of 40 mg kg^{-1} s.c., 30 μg/mouse i.c.v., 60 mg kg^{-1} p.o. or 30 mg kg^{-1} i.v., and both enantiomers at the dose of 30 mg kg^{-1} s.c. was not impaired compared with controls (table 1). On the contrary, (±)-PG-9 administered at higher doses (50 and 60 mg kg^{-1} s.c., 40 μg/mouse i.c.v., 80 mg kg^{-1} p.o. or 50 mg kg^{-1} i.v.) as well as *R*-(+)-PG-9 (40 mg kg^{-1} s.c.) and *S*-(−)-PG-9 (40 mg kg^{-1} s.c.) significantly impaired the rota-rod performance (table 1). The number of falls by control animals progressively decreased at every measurement because the mice learned how to balance on the rotating rod. The spontaneous motility of mice was not modified by treatment with (±)-PG-9 (30 and 40 mg kg^{-1} s.c.) as revealed by the Animex apparatus (data not shown).

***In vitro* functional studies.** (±)-PG-9 blocked the McN-A-343-induced inhibition of twitch contractions of the rabbit vas deferens (pK_B = 6.71 ± 0.05), antagonized the negative inotropic carbachol-induced effect in the guinea pig left atrium (pK_B = 6.85 ± 0.10), the contractile responses to acetylcholine in guinea pig ileum (pK_B = 6.84 ± 0.08) and to carbachol in immature guinea pig uterus (pK_B = 7.72 ± 0.05) as shown in table 2. Increasing concentrations of (±)-PG-9 produced parallel shifts of the agonist concentration-response curves progressively to the right and no appreciable

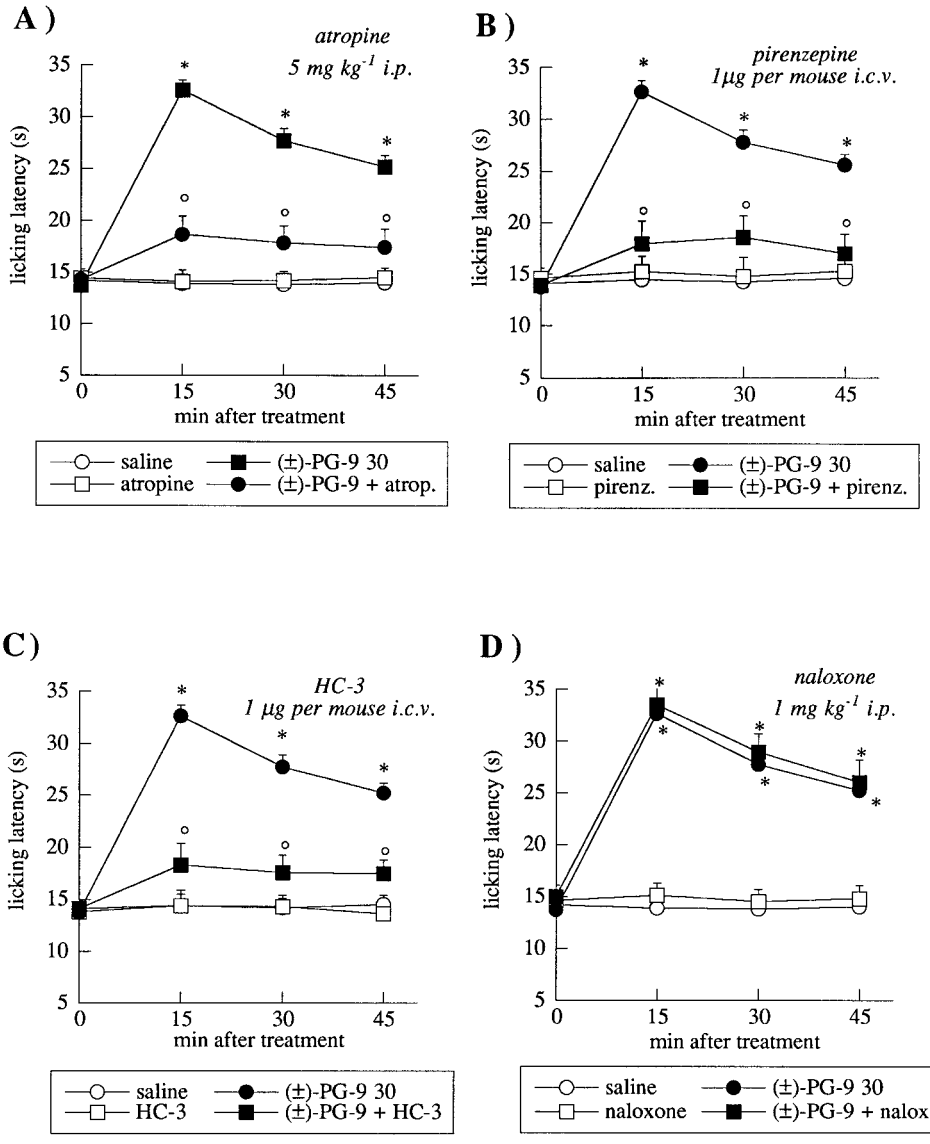

Fig. 8. Effect of atropine (5 mg kg^{-1} i.p.; A), pirenzepine (0.1 µg/mouse i.c.v.; B), HC-3 (1 µg/mouse i.c.v.; C) and naloxone (1 mg kg^{-1} i.p.; D) on antinociception induced by (±)-PG-9 in the mouse hot-plate test. Atropine, pirenzepine, HC-3 and naloxone were injected, respectively, 15 min, 5 min, 5 h and 15 min before (±)-PG-9 administration. Vertical lines show S.E.M. *P < .01 in comparison with saline controls; °P < .01 in comparison with (±)-PG-9 (30 mg kg^{-1} s.c.). Each point represents the mean of at least 10 mice. The doses of (±)-PG-9 are expressed as milligrams per kilogram s.c.

change in basal tension or maximum agonist response was observed (data not shown). pA_2 values of *R*-(+)-hyoscyamine and AFDX-116, used as reference drugs, are shown in table 2. The selectivity ratios for (±)-PG-9, *R*-(+)-hyoscyamine and AFDX-116, obtained as differences between respectively pK_B or pA_2 values, are reported in table 2.

Finally, (±)-PG-9 was shown to be endowed with weak antiacetylcholinesterase activity in comparison with physostigmine (IC_{50} $1.2 \cdot 10^{-8}$ M), its IC_{50} value on electrical eel acetylcholinesterase being $1.5 \cdot 10^{-4}$ M (data not shown).

Discussion

(±)-PG-9 was able to induce antinociception in mice, rats and guinea pigs and to prevent impairment of the acquisition of a passive-avoidance response induced by antimuscarinic drugs. Antinociception was elicited regardless of which noxious stimulus was used: thermal (hot-plate and tail-flick tests), chemical (abdominal-constriction test) and mechanical (paw-pressure test). (±)-PG-9 antinociception was obtained without visibly modifying animal gross behavior. Moreover, (±)-PG-9-treated mice showed a complete integrity of motor coordination in the rota-rod test, as well as normal spontaneous motility as revealed by the Animex test.

(±)-PG-9 antinociception was prevented by the nonselective muscarinic antagonist atropine, the M_1-antagonists pirenzepine and dicyclomine and the ACh depletor HC-3, demonstrating, like *R*-(+)-hyoscyamine (see introduction), antinociceptive properties underlying a cholinergic mechanism. Both enantiomers of PG-9, *R*-(+) and *S*-(−), contrary to atropine in which the analgesic activity resides only in the *R*-(+)-isomer (Ghelardini *et al.*, 1992), showed very similar antinociceptive properties in the presence of either a thermal or chemical stimulus. However, in both analgesic tests used, *R*-(+)-PG-9 was more effective than *S*-(−)-PG-9 even if the statistical significance was not reached. Furthermore, (±)-PG-9 showed greater efficacy than that exerted by *R*-(+)-hyoscyamine. The analgesic effect of (±)-PG-9 was also compared with the analgesia induced by some analgesic drugs such as morphine, diphenhydramine and clomipramine at the highest doses that did not impair the rota-rod performances. By comparing the areas under the curve, the antinociceptive efficacy of (±)-PG-9 (30 mg kg^{-1} s.c.) was almost

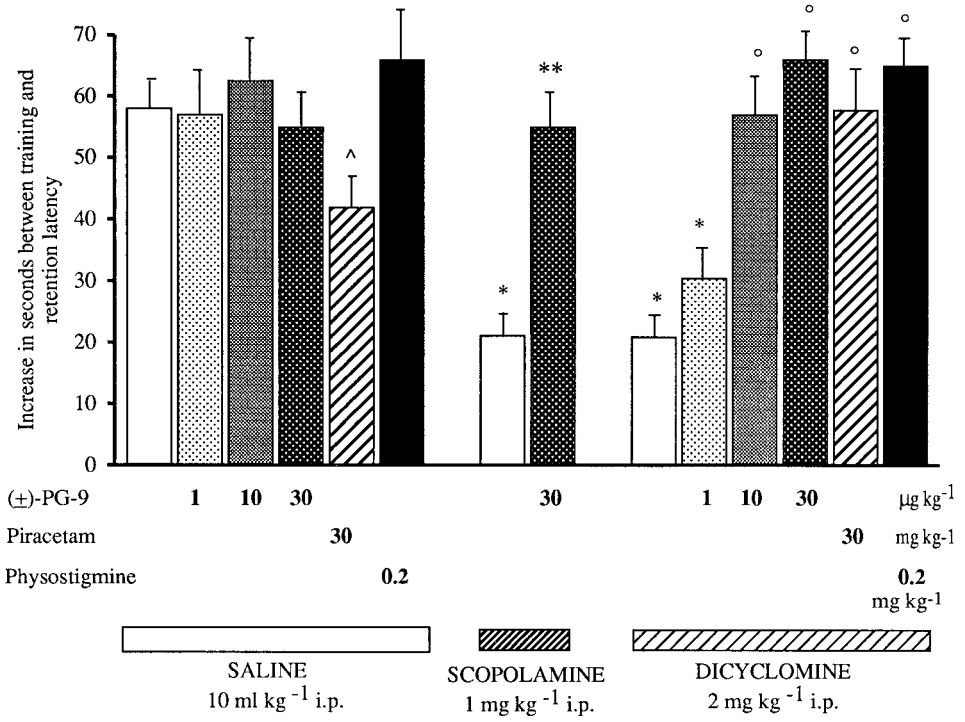

Fig. 9. Effect of i.p. (±)-PG-9 in comparison with piracetam and physostigmine on scopolamine and dicyclomine induced amnesia in mouse passive-avoidance test. Punishment consists of a fall into cold water. (±)-PG-9, piracetam and physostigmine were injected 20 min before the training session. Scopolamine and dicyclomine were injected immediately after the training session. $P < .05$; $^{*}P < .01$ in comparison with saline controls; $^{\circ}P < .01$ and $^{**}P < .01$ in comparison with dicyclomine- and scopolamine-treated mice, respectively. Each column represents the mean of at least 29 mice.

equal to that exerted by morphine (8 mg kg^{-1} s.c.), but was greater than that induced by diphenhydramine (20 mg kg^{-1} s.c.) and clomipramine (20 mg kg^{-1} s.c.).

Other neurotransmitter systems, such as opioid, GABAergic, catecholaminergic, serotoninergic and histaminergic, are not involved in (±)-PG-9 antinociception because the opioid antagonist naloxone, the $GABA_B$ antagonist CGP-35348 and the polyamine depletor reserpine, were all unable to prevent the effect of (±)-PG-9. The doses and administration schedules of the above-mentioned drugs were ideal for preventing antinociception induced by morphine (Ghelardini *et al.*, 1992), the $GABA_B$ agonist baclofen (Malcangio *et al.*, 1991) and the antidepressant drugs clomipramine and amitriptyline (Galeotti *et al.*, 1995), respectively.

(±)-PG-9 exerted its antinociceptive effect by acting centrally. In fact, it was possible to reach the same intensity of analgesia by injecting directly into the cerebral ventricles doses (10–30 μg/mouse) of (±)-PG-9 which were 50 times lower than those needed parenterally. Dependence of the antinociception on a retrodiffusion of the drug from the cerebral ventricles to the periphery can thus be ruled out.

The prevention by the i.c.v. injection of the M_1-antagonist pirenzepine and the ACh depletor HC-3 further supports the hypothesis of a central cholinergic mechanism for (±)-PG-9 antinociception and indicates a presynaptic facilitation of cholinergic transmission by (±)-PG-9. A postsynaptic mechanism of action can be ruled out because HC-3 can not antagonize antinociception induced by agonists of postsynaptic muscarinic receptors such as oxotremorine, McN-A-343 and AF-102B (Bartolini *et al.*, 1987, 1992).

The hypothesis of a presynaptic cholinergic mechanism for (±)-PG-9 agrees with previous results that demonstrate, by microdialysis studies, an increase in ACh release from rat cerebral cortex induced by both *R*-(+)-PG-9 and *S*-(−)-PG-9 administration (Romanelli *et al.*, 1995). This effect occurred in the same range of doses in which the above-mentioned compound exerted its antinociceptive activity. Because ACh release can be increased by blocking M_2/M_4 muscarinic autoreceptors (Lapchak *et al.*, 1989; Töröcsik and Vizi, 1991; McKinney *et al.*, 1993; Stillman *et al.*, 1993) and because *R*-(+)-hyoscyamine not only increased ACh release (Ghelardini *et al.*, 1997) but also showed a very high affinity for the prepuberal guinea pig uterus putative M_4 receptors (Ghelardini *et al.*, 1993), the (±)-PG-9 affinity profile toward muscarinic receptor subtypes was investigated *in vitro*. The affinity profile of (±)-PG-9 *versus* M_1 (rabbit vas deferens), M_2 (guinea pig atrium), M_3 (guinea pig ileum) and putative M_4 receptors (prepuberal guinea pig uterus) was evaluated by *in vitro* functional studies. Because *R*-(+) and *S*-(−)-PG-9 were not endowed with a different analgesic profile, their *in vitro* selectivity toward the muscarinic receptor subtypes was not considered worth investigating. The M_4 muscarinic receptor subtype has been defined as putative because it has not been confirmed that the mRNA codifying M_4 is expressed in prepuberal uterine tissue. However, pharmacological and biochemical studies show that the M_4 putative receptor of prepuberal guinea pig uterus has a pharmacological and biochemical profile identical with that of the muscarinic M_4 receptor subtype expressed in the rat striatum (McKinney *et al.*, 1991; Waelbroeck *et al.*, 1992) and in NG 108–15 cells (Leiber *et al.*, 1984; Marc *et al.*, 1986). (±)-PG-9 showed, like *R*-(+)-hyoscyamine, a M_4/M_1 muscarinic receptor subtype selectivity ratio (10.2 times) higher than the M_2/M_1 selectivity ratio (1.4 times).

The antinociception induced by (±)-PG-9 may be caused by the antagonism of the M_4 muscarinic autoreceptor. The selectivity on blocking the M_2/M_4 toward M_1 was evaluated because Bartolini *et al.* (1992) demonstrated that the muscarinic postsynaptic receptor responsible for central cholinergic antinociception belongs to the M_1 subtype.

The antinociceptive efficacy of (±)-PG-9 was greater than that of *R*-(+)-hyoscyamine. (±)-PG-9 is also endowed with

TABLE 1
Effect of (±)-PG-9, *R*-(+)-PG-9 and *S*-(−)-PG-9 in the mouse rota-rod test

Treatment	Dose	Number of falls in 30 s[a]			
		Before pretreatment	After treatment		
			15 min	30 min	45 min
Saline	10 ml · kg^{-1} s.c.	3.5 ± 0.3	2.4 ± 0.3**	1.6 ± 0.2**	1.2 ± 0.2**
(±)-PG-9	40 mg · kg^{-1} s.c.	3.2 ± 0.4	1.8 ± 0.3**	1.5 ± 0.3**	0.8 ± 0.2**
(±)-PG-9	50 mg · kg^{-1} s.c.	3.0 ± 0.4	3.3 ± 0.4*	2.9 ± 0.4*	2.1 ± 0.4*
(±)-PG-9	60 mg · kg^{-1} s.c.	2.9 ± 0.4	3.6 ± 0.5*	3.5 ± 0.4*	2.6 ± 0.3*
R-(+)-PG-9	30 mg · kg^{-1} s.c.	3.2 ± 0.3	1.7 ± 0.2**	1.1 ± 0.3**	0.9 ± 0.2**
R-(+)-PG-9	40 mg · kg^{-1} s.c.	3.0 ± 0.4	3.2 ± 0.4*	3.5 ± 0.5*	2.5 ± 0.4*
S-(−)-PG-9	30 mg · kg^{-1} s.c.	3.4 ± 0.3	1.6 ± 0.3**	1.1 ± 0.3**	0.7 ± 0.2**
S-(−)-PG-9	40 mg · kg^{-1} s.c.	3.0 ± 0.4	3.3 ± 0.4*	3.4 ± 0.4*	2.6 ± 0.3*
Saline	5 μl i.c.v.	3.4 ± 0.4	1.6 ± 0.3**	1.1 ± 0.3**	0.8 ± 0.2**
(±)-PG-9	30 μg i.c.v.	3.1 ± 0.4	2.0 ± 0.4**	0.8 ± 0.3**	0.8 ± 0.3**
(±)-PG-9	40 μg i.c.v.	2.8 ± 0.4	3.1 ± 0.3*	3.0 ± 0.4*	2.3 ± 0.4*
Saline	10 ml · kg^{-1} p.o.	3.2 ± 0.3	1.9 ± 0.3**	1.0 ± 0.2**	0.8 ± 0.2**
(±)-PG-9	60 mg · kg^{-1} p.o.	2.9 ± 0.4	1.3 ± 0.4**	1.2 ± 0.3**	0.7 ± 0.2**
(±)-PG-9	80 mg · kg^{-1} p.o.	3.2 ± 0.4	3.3 ± 0.4*	2.7 ± 0.3*	1.6 ± 0.2*
Saline	10 ml · kg^{-1} i.v.	3.1 ± 0.4	2.2 ± 0.4**	1.5 ± 0.3**	1.1 ± 0.3**
(±)-PG-9	30 ml · kg^{-1} i.v.	3.3 ± 0.4	2.3 ± 0.4**	1.1 ± 0.3**	0.8 ± 0.3**
(±)-PG-9	50 ml · kg^{-1} i.v.	2.8 ± 0.2	3.4 ± 0.4*	3.1 ± 0.3*	2.8 ± 0.3*

[a] Each value represents the mean of 5 to 10 mice.
* $P < .01$ in comparison with saline controls.
** $P < .01$ in comparison with its pretest.

TABLE 2
Affinity profiles of (±)-PG-9, *R*-(+)-hyoscyamine and AFDX-116 at muscarinic M_1-receptors in rabbit vas deferens, M_2-receptors in guinea-pig left atrium, M_3-receptors in guinea-pig ileum and M_4 putative-receptors in guinea-pig uterus[a]

Drug	pA_2 values				Selectivity ratios		
	M_1 rabbit vas deferens	M_2 guinea pig left atrium	M_3 guinea pig ileum	M_4-putative guinea pig uterus	M_4/M_1	M_2/M_1	M_4/M_2
(±)-PG-9	6.71 ± 0.05[b]	6.85 ± 0.10[b]	6.84 ± 0.08[b]	7.72 ± 0.05[b]	10.2	1.4	7.4
R-(+)-Hyoscyamine	7.05 ± 0.05[c]	7.25 ± 0.04[c]	6.88 ± 0.05[c]	9.56 ± 0.01[c]	323	1.6	204
AFDX-116	6.84 ± 0.14[d]	7.12 ± 0.11[d]	6.34 ± 0.13[e]	6.70 ± 0.06	0.7	1.9	0.4

[a] The ratios of affinity constants are given as a measure of receptor selectivity.
[b] pK_B values are obtained with 1 μM (±)-PG-9 with the exception of the guinea pig uterus (0.1 μM). Each value is the mean of at least five experiments. pA_2 values are the mean ± S.E.M. of 12 to 16 preparations. Selectivity ratios were calculated as antilogs of the difference between pK_B or pA_2 values. Agonists used: McN-A-343 (rabbit vas deferens), carbachol (guinea pig left atrium and uterus) and ACh (guinea pig ileum) (see "Methods").
[c] Ghelardini *et al.* (1993).
[d] Eltze (1988).
[e] Eltze and Figala (1988).

very low anticholinesterase activity as demonstrated by the *in vitro* evaluation of its IC_{50} value ($IC_{50} = 1.5 \cdot 10^{-4}$ M). It is possible that (±)-PG-9 is able to amplify cholinergic neurotransmission through the antagonism of the muscarinic autoreceptor and that this effect, in turn, is potentiated by its low cholinesterase inhibitory activity. However, we cannot exclude that other mechanisms able to potentiate the endogenous cholinergic system may be involved in the antinociception induced by (±)-PG-9.

D_2 dopaminergic (Gorell and Czarnecki, 1986; Wedzony *et al.*, 1988; Scatton, 1992; Imperato *et al.*, 1993), H_3 histaminergic (Clapham and Kilpatrick, 1992), 5-HT_4 serotoninergic heteroreceptors (Consolo *et al.*, 1994), all located on central cholinergic neurons, increase ACh release. Therefore, the involvement of the above-mentioned heteroreceptors was investigated. Quinpirole (D_2 agonist), *R*-(α)-methylhistamine (H_3 agonist) and SDZ-205557 (5-HT_4 antagonist), at doses able to prevent antinociception induced respectively by haloperidol (Ghelardini *et al.*, 1992), thioperamide (Malmberg-Aiello *et al.*, 1994), BIMU 1 and BIMU 8 (Ghelardini *et al.*, 1996), failed to prevent (±)-PG-9 antinociception. It has also been observed that the activation of the serotoninergic autoreceptor 5-HT_{1A} enhances ACh release from the guinea pig cortex (Bianchi *et al.*, 1990). Pretreatment with the 5-HT_{1A} selective antagonist NAN 190 at doses which block the antinociception induced by 5-HT_{1A} agonists (Ghelardini *et al.*, 1994), did not prevent the enhancement of the pain threshold produced by (±)-PG-9 administration. The present data suggest that the above-mentioned receptors, even though they are able to increase ACh release, are not involved in (±)-PG-9 mechanism of analgesic action.

(±)-PG-9 was able to prevent impairment of the acquisition of a passive-avoidance response induced by the antimuscarinic drugs scopolamine and dicyclomine in mice. Because stimulation of the cholinergic system improves cognitive processes (Coyle, 1995), it is reasonable to suppose that the antiamnesic effect induced by (±)-PG-9 could be related to its ability to activate the cholinergic system. In our experimental conditions, (±)-PG-9 was administered before mice received the aversive stimulus in correspondence to the maximum analgesic effect. The ability of (±)-PG-9 to enhance the pain threshold by abolishing the perception of the punishing stimulus may have influenced the results obtained. An electric shock, reported as the punishing stimulus in the original method (Jarvik and Kopp, 1967), was thus substituted by a nonpainful stimulus consisting of a fall into cold water.

In summary, our results have shown that (±)-PG-9 is able to produce dose-dependent antinociception in rodents and guinea pigs as well as antiamnesic activity in mice, without impairing motor coordination, by potentiating endogenous cholinergic activity.

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Send reprint requests to: Dr. Carla Ghelardini, Department of Pharmacology, University of Florence, Viale G.B. Morgagni, 65, I-50134 Florence, Italy.