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

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

Accepted for publication November 6, 1997 This paper is available online at http://www.jpet.org

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 i.p.; 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 M1-selective antagonists pirenzepine and dicyclomine and the acetylcholine depletor hemicholinium-3, but not by the opioid antagonist naltrexone, the γ-amino butyric acid antagonist 3-amino propyl diethoxy methyl-phosphonic acid, the H3 agonist R-(α)-methylhistamine, the D2 antagonist quinpirole, the 5-hydroxytryptamine2 antagonist 2-methoxy-4-amino-5-chlorobenzoic acid 2-(diethylamino)ethyl ester hydrochloride, the 5-hydroxytryptamine1A 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 M1, guinea pig atrium for M2, guinea pig ileum for M3 and immature guinea pig uterus for putative M4), have shown an M4/M1 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 M1 receptors. These authors reported that M1-selective agonists McN-A-343 and AF-102B were able to produce a significant enhancement of the pain threshold, whereas the M2-selective agonist arecaidine propargil ester (APE) was not. Moreover, Bartolini et al. (1992) have demonstrated that the M1 antagonists dicyclomine and pirenzepine, contrary to the M2 antagonist AF-DX 116, antagonized antinociception induced by both unselective (oxotremorine) and M1-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 confirms 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, salivation, 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(−)-

ABBREVIATIONS: i.c.v., intracerebroventricular; s.c., subcutaneous; i.p., intraperitoneal; p.o., per os; i.v., intravenous; PG-9, 3α-tropyl 2-(p-bromophenyl)propionate; McN-A-343, 4-[(3-chlorophenyl)-carbamoyloxy]-2-butyltrimethylammonium chloride; AF-DX 116, 11.2-(diethylamino)methyl-1-piperidinyl acetate-5,11-dihydro-6H-pyrido 2,3-b 1,4 benzodiazepine-6-one; CGP 35348, 3-aminopropyl diethoxy methyl-phosphonic acid; RAMH, (R)-α-methylhistamine; NAN-190, 1-(2-methoxyphenyl)-4-[4-(2-phthalimido)butyl]piperazine hydrobromide; SDZ 205567, 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-amino butyric acid.
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, \( \pm \)-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, etc.)...
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 \( R-(+)-\text{hyoscymine} \) was able to prevent amnesia induced by both scopolamine and dicyclomine in mice (Ghelardini et al., 1997), the potential anti-amnesic activity of \((\pm)-\text{PG-9}\) 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.
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
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 calculated graphically for the calculation of dose ratios.

Determination of antagonist affinities. After a stabilization period 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 EC50 values in the absence and presence of antagonists were determined graphically.

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-5, pirenzepine dihydrochloride, naloxone hydrochloride, quinpirole hydrochloride, (R)-α-methylhistamine dihydro-
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 \( R(-)-\)hyoscymine 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 McMornick (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-\(\mu\)l 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 \(\mu\)l (mice) or 10 \(\mu\)l (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 \(\mu\)l 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 \(\mu\)g/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...
Antagonism of the $(\pm)$-PG-9 induced antinociception. In the mouse hot-plate test, the antinociceptive effect of $(\pm)$-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 $\mu$mouse i.c.v.), $(R)$-o-methylhistamine (10 mg kg$^{-1}$ i.p.), quinpirole (0.1 mg kg$^{-1}$ i.p.), SDZ-205557 (10 mg kg$^{-1}$ i.p.), NAM 190 (0.5 $\mu$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 $\mu$mouse i.c.v.) and hemicholinium-3 (1 $\mu$mouse or rat i.c.v.) were able to completely prevent $(\pm)$-PG-9 antinociception in the mouse hot-plate (fig. 8, A–C), abdominal-constriction (fig. 3A) and rat paw-pressure tests (figs. 4, C and D). All antagonists were injected 15 min before $(\pm)$-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 $(\pm)$-PG-9).

Antiamnesic activity of $(\pm)$-PG-9. Pretreatment with $(\pm)$-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. $(\pm)$-PG-9 enhanced the entrance latency up to a value similar to that produced by control animals (fig. 9). $(\pm)$-PG-9, at 1 mg kg$^{-1}$ i.p., was completely ineffective (fig. 9). The antiamnesic effect of $(\pm)$-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.).

$(\pm)$-PG-9, when given alone, at the highest doses used, had no effect on the mouse passive-avoidance test in comparisons 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 $(\pm)$-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 $(\pm)$-PG-9 at the dose of 40 mg kg$^{-1}$ s.c., 30 mg 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, $(\pm)$-PG-9 administered at higher doses (50 and 60 mg kg$^{-1}$ s.c., 40 $\mu$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 $(\pm)$-PG-9 (30 and 40 mg kg$^{-1}$ s.c.) as revealed by the Animex apparatus (data not shown).

In vitro functional studies. $(\pm)$-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 $(\pm)$-PG-9 produced parallel shifts of the agonist concentration-response curves progressively to the right and no appreciable
change in basal tension or maximum agonist response was observed (data not shown). pA2 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 pKB or pA2 values, are reported in table 2.

Finally, (±)-PG-9 was shown to be endowed with weak antiacetylcholinesterase activity in comparison with physostigmine (IC50 1.2 × 10−2 M), its IC50 value on electrical eel acetylcholinesterase being 1.5 × 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 M1-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...
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$_1$/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., 1992), 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$_4$ (guinea pig atrium), M$_6$ (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$_4$/M$_2$ 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$_4$/M$_1$ toward M$_4$ was evaluated because Bartolini et al. (1992) demonstrated that the muscarinic postsynaptic receptor responsible for central cholinergic antinociception belongs to the M$_4$ 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 rotarod test

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose</th>
<th>Number of falls in 30 s**</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Before pretreatment</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15 min</td>
</tr>
<tr>
<td>Saline</td>
<td>10 ml · kg⁻¹ i.c.</td>
<td>3.5 ± 0.3</td>
</tr>
<tr>
<td>(+)-PG-9</td>
<td>40 mg · kg⁻¹ i.c.</td>
<td>3.2 ± 0.4</td>
</tr>
<tr>
<td>(+)-PG-9</td>
<td>50 mg · kg⁻¹ i.c.</td>
<td>3.0 ± 0.4</td>
</tr>
<tr>
<td>(+)-PG-9</td>
<td>60 mg · kg⁻¹ i.c.</td>
<td>2.9 ± 0.4</td>
</tr>
<tr>
<td>R-(-)-PG-9</td>
<td>30 mg · kg⁻¹ i.c.</td>
<td>3.2 ± 0.3</td>
</tr>
<tr>
<td>(+)-PG-9</td>
<td>40 mg · kg⁻¹ i.c.</td>
<td>3.0 ± 0.4</td>
</tr>
<tr>
<td>S-(−)-PG-9</td>
<td>30 mg · kg⁻¹ i.c.</td>
<td>3.4 ± 0.3</td>
</tr>
<tr>
<td>S-(−)-PG-9</td>
<td>40 mg · kg⁻¹ i.c.</td>
<td>3.0 ± 0.4</td>
</tr>
</tbody>
</table>

| 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 ± 0.3 | 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 pretreatment.

TABLE 2
Affinity profiles of (+)-PG-9, R-(-)-hyoscyamine and AFDX-116 at muscarinic M₁ receptors in rabbit vas deferens, M₃ receptors in guinea-pig left atrium, M₄ receptors in guinea-pig ileum and M₅ putative-receptors in guinea-pig uterus

<table>
<thead>
<tr>
<th>Drug</th>
<th>¹</th>
<th>²</th>
<th>³</th>
<th>⁴</th>
<th>⁵</th>
<th>⁶</th>
<th>Selectivity ratios</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M₁ rabbit vas deferens</td>
<td>M₂ guinea pig left atrium</td>
<td>M₃ guinea pig ileum</td>
<td>M₄ guinea pig uterus</td>
<td>M₅ putative guinea pig uterus</td>
<td>M₅/M₁</td>
<td>M₄/M₁</td>
</tr>
<tr>
<td>(+)-PG-9</td>
<td>6.71 ± 0.05⁷</td>
<td>6.85 ± 0.10⁸</td>
<td>6.84 ± 0.08⁸</td>
<td>7.72 ± 0.05⁹</td>
<td>10.2</td>
<td>1.4</td>
<td>7.4</td>
</tr>
<tr>
<td>R-(-)-Hyoscyamine</td>
<td>7.05 ± 0.05⁰</td>
<td>7.25 ± 0.04⁴</td>
<td>6.88 ± 0.05⁶</td>
<td>9.56 ± 0.01⁷</td>
<td>323</td>
<td>1.6</td>
<td>204</td>
</tr>
<tr>
<td>AFDX-116</td>
<td>6.84 ± 0.14¹</td>
<td>7.12 ± 0.11¹</td>
<td>6.34 ± 0.13¹</td>
<td>6.70 ± 0.06³</td>
<td>0.7</td>
<td>1.9</td>
<td>0.4</td>
</tr>
</tbody>
</table>

¹ The ratios of affinity constants are given as a measure of receptor selectivity.
² pKᵦ 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. pA₂ values are the mean ± S.E.M. of 12 to 16 preparations. Selectivity ratios were calculated as antilogs of the difference between pKᵦ or pA₂ values. Agonists used: McN-A-343 (rabbit vas deferens), carbachol (guinea pig left atrium and uterus) and ACh (guinea pig ileum) (see “Methods”).
³ Ghelardini et al. (1993).
⁴ Eltze (1988).
⁵ Eltze and Figala (1988).

very low anticholinesterase activity as demonstrated by the in vitro evaluation of its IC₅₀ value (IC₅₀ = 1.5·10⁻⁴ 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 antinoceptive induction by (+)-PG-9.

D₂ dopaminergic (Gorell and Czarnecki, 1986; Wedzony et al., 1988; Scatton, 1992; Imperato et al., 1993), H₂ histaminergic (Clapham and Kilpatrick, 1992), 5-HT₄ 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 (D₂ agonist), R-(α)-methylhistamine (H₂ agonist) and SDZ-205557 (5-HT₄ antagonist), at doses able to prevent antinoicence 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 antinoice. It has also been observed that the activation of the serotoninergic autoreceptor 5-HT₁₄ enhances ACh release from the guinea pig cortex (Bianchi et al., 1990). Pretreatment with the 5-HT₁₄ selective antagonist NAN 190 at doses which block the antinoicence induced by 5-HT₁₄ 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 antimuscarnic drugs scopolamine and dicyclomine in mice. Because stimulation of the cholinergic system improves cognitive processes (Coyle, 1995), it is reasonable to suppose that the antiaimnesic 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 antinoicence 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 (+)-PD 9-is able to produce dose-dependent antinociception in rodents and guinea pigs as well as anti-anaesthetic activity in mice, without impairing motor coordination, by potentiating endogenous cholinergic activity.

Acknowledgment

The authors thank Ciba Geigy for the gift of CGP-35348.

References


Clapham J and Kilpatrick GJ (1992) Histamine H3 receptors modulate the release of cholinergic activity. Impairing motor coordination, by potentiating endogenous 


Elze M (1988) Muscarinic 


Goldberg MJ and Kupferman ME (1995) 5-HT3 receptors modulate the release of cholinergic activity. Impairing motor coordination, by potentiating endogenous 


Jendel DJ, Goldberg MJ and Kupferman ME (1995) 5-HT3 receptors modulate the release of cholinergic activity. Impairing motor coordination, by potentiating endogenous 


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