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ANTIHISTAMINE ANTINOCICEPTION IS MEDIATED BY Gi-PROTEIN ACTIVATION

N. GALEOTTI, C. GHELARDINI* and A. BARTOLINI

Department of Preclinical and Clinical Pharmacology, Viale G. Pieraccini 6, I-50139 Florence, Italy

Abstract—The effect of the i.c.v. administration of antisense oligodeoxynucleotides directed against the α subunit of different Gi-proteins (anti-Giα_1, anti-Giα_2, anti-Giα_3) on the antinociception induced by the H_1-antihistamines was evaluated in the mouse hot-plate test. The administration of diphenhydramine (20 mg kg^{-1} s.c.), pyrilamine (15 mg kg^{-1} s.c.) and promethazine (6 mg kg^{-1} s.c.) produced an increase of the pain threshold which peaked 15 min after injection. Pretreatment with anti-Giα_1 (12.5 μg per mouse i.c.v.), anti-Giα_2 (25 μg per mouse i.c.v.) and anti-Giα_3 (25 μg per mouse i.c.v.), administered 24 and 18 h before test, prevented the antihistamine-induced antinociception. At the highest effective doses, none of the compounds used impaired motor coordination, as revealed by the rota rod test, nor modified spontaneous motility and inspection activity, as revealed by the hole board test.

These results suggest an important role played by the Gi-protein pathway in the transduction mechanism involved in the enhancement of the pain threshold produced by H_1-antihistamines.

Key words: Gi-proteins, pain, H_1-antihistamines, diphenhydramine, pyrilamine, promethazine.

The histamine H_1-receptor antagonists are among the most widely used medications in the world. These compounds produce the inhibition of the effects of histamine mediated by H_1 receptors such as smooth-muscle contraction in the respiratory and gastrointestinal tracts, pruritus, sneezing by sensory-nerve stimulation, vasodilation (Simons and Simons, 1994). Therefore, their most common use is in the treatment of allergic disorders (Rimer and Church, 1990). In addition to these well-known peripheral effects, H_1-receptor antagonists produce various central inhibitory actions (Simons and Simons, 1994). Antihistamines are widely used as adjuvants in preoperative analgesia as well as in postoperative pain. These compounds decrease the amount of narcotic that is necessary, provides sedating, and other antihistaminic effects that are helpful in certain clinical situations (Hupert et al., 1980; Sunshine et al., 1989). H_1-antagonists are also able to potentiate opioid analgesia in laboratory animals (Sun et al., 1985; Yeh, 1985; Malec, 1987). It has also been observed that some antihistamines, such as hydroxyzine, diphenhydramine, pyrilamine and promethazine, are endowed with analgesic properties in both laboratory animals (Stambaugh and Lane, 1983; Rumore and Schlichting, 1985; Sun et al., 1985; Rumore and Schlichting, 1986) and humans (Campos and Solis, 1980; Rumore and Schlichting, 1986).

Even if the analgesic properties of antihistamines are well established, little is known about the intracellular mechanism of action of these compounds. Recently, it has been reported that the antinociception induced by H_1-receptor antagonists underlies the activation of a signal transduction mechanism operated by Gi-proteins. The i.c.v. administration of pertussis toxin (PTX), compound which selectively inactivates Gi-proteins, prevented the increase of the pain threshold induced by diphenhydramine, pyrilamine and promethazine in the mouse hot-plate test (Galeotti et al., 1996, 1999). Gi-proteins represent the most widespread modulatory signalling pathway in neurones (Holz et al., 1986) and their involvement in the modulation of pain perception has been determined. The administration of PTX produced hyperalgesia and allodynia in laboratory animals (Ohnishi et al., 1990; Galeotti et al., 1996; Womer et al., 1997). A reduction of pain perception has also been observed in knock-out mice lacking for the gene encoding the subtype Go of the Gi-protein family (Jiang et al., 1998). These data clearly indicate that a lack of functionality of Gi-proteins enhances the sensitivity to pain.

To elucidate the intracellular effectors involved in the mechanism of action of antihistamines, we decided to further investigate the role of Gi-proteins in the diphenhydramine-, pyrilamine- and promethazine-induced enhancement of the pain threshold by using an antisense strategy. Antisense oligodeoxynucleotides (aODN) are short synthetic DNA segments complementary to sequences of an mRNA target. By forming DNA/mRNA heteroduplexes, aODNs can transiently inacti-

*Corresponding author. Tel.: +39-55-4271312; fax: +39-55-4271280.

E-mail address: ghelard@server1.pharm.unifi.it (C. Ghelardini).

Abbreviations: aODN, antisense oligodeoxynucleotide; dODN, degenerate oligodeoxynucleotide; PTX, pertussis toxin.

vate single genes. Since several G-protein subtypes belong to the Gi family, in the present study we used aODN against the α subunits of the Gi_1, Gi_2 and Gi_3 proteins in order to determine the role of each subtype in the antinociception induced by the above-mentioned antihistamines.

EXPERIMENTAL PROCEDURES

Animals

Male Swiss albino mice (23–30 g) from the Morini (San Polo d'Enza, Italy) breeding farm were used. Fifteen mice were housed per cage. The cages were placed in the experimental room 24 h before the test for acclimatisation. The animals were fed a standard laboratory diet and tap water *ad libitum* and kept at 23 ± 1°C with a 12-h light/dark cycle, light on at 07.00 h. All experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) for experimental animal care. All efforts were made to minimise the number of animals used and their suffering.

I.c.v. injection technique

I.c.v. administration was performed under ether anaesthesia, according to the method described by Haley and McCormick (1957). In brief, during anaesthesia, mice were grasped firmly by the loose skin behind the head. A 0.4-mm external diameter hypodermic needle attached to a 10-μl syringe was inserted perpendicularly through the skull and no more than 2 mm into the brain of the mouse, where 5 μl were then administered. The injection site was 1 mm to the right or left from the midpoint on a line drawn through to the anterior base of the ears. Injections were performed into the right or left ventricle, randomly. To ascertain that the drugs were administered exactly into the cerebral ventricle, some mice (20%) were injected with 5 μl of diluted 1:10 India ink and their brains examined macroscopically after sectioning. The accuracy of the injection technique was evaluated and the percentage of correct injections was 95.

Hot-plate test

The method adopted was described by O'Callaghan and Holtzman (1975). Mice were placed inside a stainless steel container, which was set thermostatically at 52.5 ± 0.1°C in a precision water-bath from KW Mechanical Workshop (Siena, Italy). Reaction times (s), were measured with a stopwatch before and 15, 30, 45 and 60 min after diphenhydramine, pyrilamine and promethazine administration. 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.

The licking latency values reported in all figures were evaluated in relation to the maximum analgesic effect of diphenhydramine, pyrilamine and promethazine, which was reached 15 min after administration.

The doses of antihistamines used in the present research resulted from data obtained by producing dose–response curves in the hot-plate and rota rod tests which indicate them as the highest effective doses devoid of behavioural impairment (Galeotti et al., 1999).

Hole board test

The hole board test was performed according to Galeotti et al. (2001). The test consisted of a 40-cm square plane with 16 flush mounted cylindrical holes (3 cm diameter) distributed four by four in an equidistant, grid-like manner. Mice were placed on the centre of the board one by one and allowed to move about freely for a period of 10 min each. Two electric eyes, crossing the plane from midpoint to midpoint of opposite sides, thus dividing the plane into four equal quadrants, automatically signalled the movement of the animal (counts in 5 min) on the surface of the plane (locomotor activity). Miniature photoelectric cells, in each of the 16 holes, recorded (counts in 5 min) the exploration of the holes (exploratory activity) by the mice. The test was performed 24–18 h after the i.c.v. injection of degenerate ODN (dODN, 25 μg per mouse) or aODN (12.5–25 μg per mouse). Twelve mice per group were tested.

Rota rod test

The apparatus consisted of a base platform and a rotating rod with a diameter of 3 cm and a non-slippery 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 on the basis of the number of falls from the rod in 30 s according to Vaught et al., (1985). Those mice scoring less than three and more than six falls in the pretest were rejected (20%). The performance time was measured before (pretest) and 15, 30 and 45 min after s.c. administration of the investigated compounds. Animals were i.c.v. pretreated 24 and 18 h prior to the test with dODN (25 μg per mouse) or aODN (12.5–25 μg per mouse). Twelve mice per group were tested.

aODN

Phosphodiester oligonucleotides (ODNs) protected from terminal phosphorothioate double substitution (capped ODNs) against possible exonuclease-mediated degradation were purchased by Tib Molbiol (Genova, Italy). The sequences of the 33-mer aODNs used in the present study were the following:

anti-$Gi_1\alpha$: 5′-G*C*T GTC CTT CCA CAG TCT CTT TAT GAC GCC G*G*C-3′
anti-$Gi_2\alpha$: 5′-A*T*G GTC AGC CCA GAG CCT CCG GAT GAC GCC C*G*A-3′
anti-$Gi_3\alpha$: 5′-G*C*C ATC TCG CCA TAA ACG TTT AAT CAC GCC T*G*C-3′

All oligos were previously characterised by *in vitro* (immunoblotting) and *in vivo* (tail flick) experiments (Raffa et al., 1994; Sanchez-Blazquez et al., 1995; Sanchez-Blazquez and Garzon, 1998). A 33-mer fully dODN 5′-N*N*N NNN NNN NNN NNN NNN NNN NNN NNN N*N*N-3′ (where N is G, or C, or A, or T) was used as a control. ODNs were vehiculated intracellularly by an artificial cationic lipid (DOTAP, Sigma) to enhance both uptake and stability, as described previously (Capaccioli et al., 1993). aODN or dODN were preincubated at 37°C for 30 min with 13 μM DOTAP and supplied to mice by i.c.v. injection of 5 μl solution 18 and 24 h prior to the behavioural tests.

Drugs

The following drugs were used: pyrilamine maleate, promethazine hydrochloride (RBI); diphenhydramine hydrochloride, D-amphetamine hydrochloride (De Angeli). Drugs were dissolved in isotonic (NaCl 0.9%) saline solution. Drug and ODNs concentrations were prepared in such a way that the necessary dose could be administered in a volume of 5 μl by i.c.v. injection or 10 ml kg^{-1} by s.c. administration.

Statistical analysis

All experimental results are given as the mean ± S.E.M. Analysis of variance (ANOVA), followed by Fisher's protected least significant difference (PLSD) procedure for post-hoc compari-

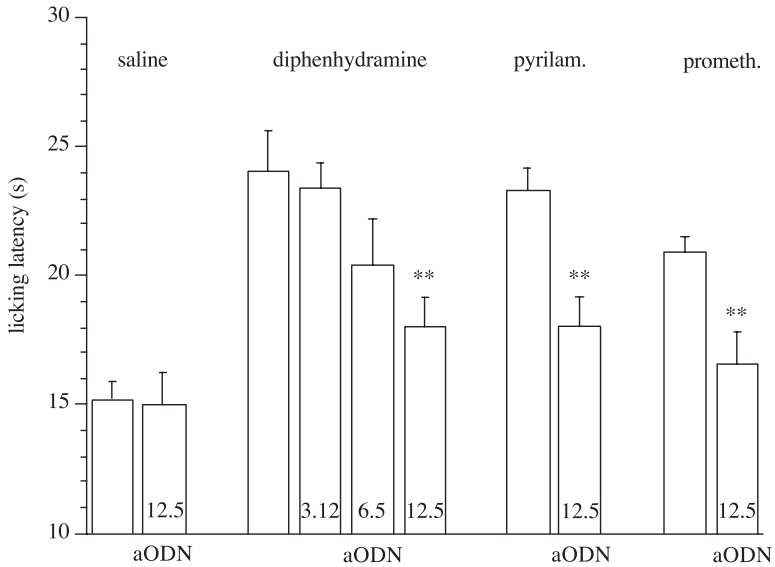

Fig. 1. Prevention by aODN against the α subunit of Gi_1 protein (3.12–12.5 μg per mouse i.c.v.) of diphenhydramine (20 mg kg^{-1} s.c.), pyrilamine (15 mg kg^{-1} s.c.) and promethazine (6 mg kg^{-1} s.c.) antinociception in the mouse hot-plate test. Vertical lines represent S.E.M. between 19 and 25 mice were tested. $^{**}P < 0.01$ in comparison with dODN-treated mice.

son, was used to verify significance between two means. Data were analysed with the StatView software for the Macintosh (1992). *P* values of less than 0.05 were considered significant.

RESULTS

Effect of aODN against Giα subunits on H_1-antihistamines antinociception

Diphenhydramine (20 mg kg^{-1} s.c.), pyrilamine (15 mg kg^{-1} s.c.) and promethazine (6 mg kg^{-1} s.c.) produced antinociception in the mouse hot-plate test (Fig. 1).

The antinociception induced by diphenhydramine (20 mg kg^{-1} s.c.) was prevented by pretreatment with the aODN against the α subunit of the Gi_1 proteins (Fig. 1). Anti-$Gi\alpha_1$ (3.12–12.5 μg per mouse i.c.v.) produced a dose-dependent antagonism of the increase of the pain threshold produced by diphenhydramine. The dose of 3.12 μg per mouse i.c.v. was completely ineffective; 6.25 μg per mouse i.c.v. partially prevented diphenhydramine antinociception, even if the statistical significance was not reached, while the doses of 12.5 μg per mouse i.c.v. reduced the licking latency up to a value comparable to that produced by control animals (Fig. 1). Pretreatment with anti-$Gi\alpha_1$ (12.5 μg per mouse i.c.v.) also prevented the antinociception induced by pyrilamine and promethazine (Fig. 1).

The administration of an aODN against the α subunit of the Gi_2 proteins (6.25–25 μg per mouse i.c.v.), dose-

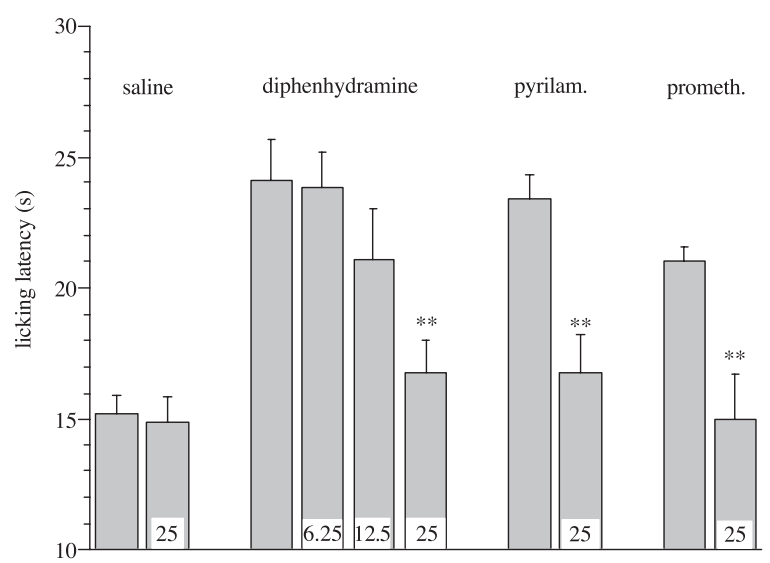

Fig. 2. Prevention by aODN against the α subunit of Gi_2 protein (6.25–25 μg per mouse i.c.v.) of diphenhydramine (20 mg kg^{-1} s.c.), pyrilamine (15 mg kg^{-1} s.c.) and promethazine (6 mg kg^{-1} s.c.) antinociception in the mouse hot-plate test. Vertical lines represent S.E.M. between 18 and 23 mice were tested. $^{**}P < 0.01$ in comparison with dODN-treated mice.

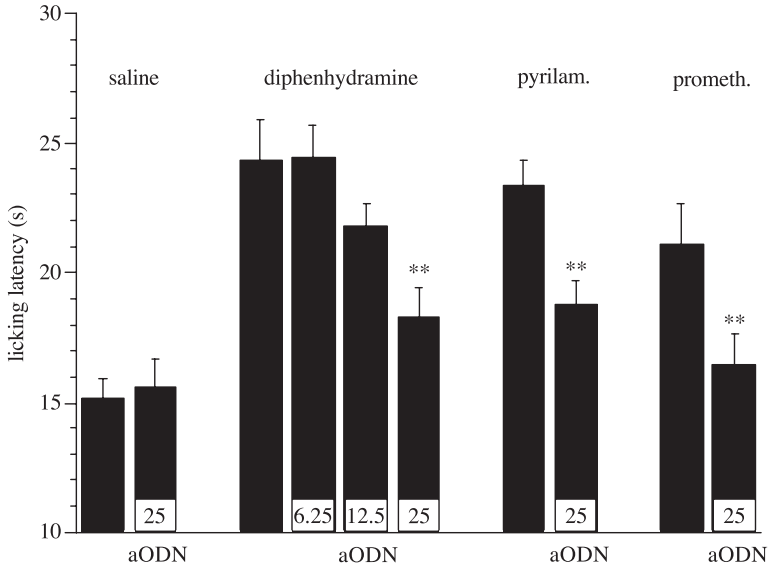

Fig. 3. Prevention by aODN against the α subunit of Gi_3 protein (6.25–25 μg per mouse i.c.v.) of diphenhydramine (20 mg kg^{-1} s.c.), pyrilamine (15 mg kg^{-1} s.c.) and promethazine (6 mg kg^{-1} s.c.) antinociception in the mouse hot-plate test. Vertical lines represent S.E.M.; between 20 and 27 mice were tested. $^{**}P < 0.01$ in comparison with dODN-treated mice.

dependently prevented diphenhydramine-induced antinociception (Fig. 2). At the concentration of 25 μg per mouse i.c.v., a complete prevention was obtained whereas, at 6.25 μg per mouse i.c.v., the aODN pretreatment was ineffective. At the highest dose employed, anti-$Gi\alpha_2$ also antagonised the increase of the pain threshold induced by both pyrilamine and promethazine (Fig. 2).

Similarly to anti-$Gi\alpha_2$ pretreatment, the administration of an aODN against the α subunit of the Gi_3 proteins (6.25–25 μg per mouse i.c.v.) antagonised, in a dose-dependent manner, the antinociception produced by diphenhydramine reaching its maximum antagonistic effect at the dose of 25 μg per mouse i.c.v. (Fig. 3). Pretreatment with anti-$Gi\alpha_3$ also prevented antinociception induced by pyrilamine and promethazine (Fig. 3).

A time-course of recovery from antihistamine antinociception after aODN treatment was performed (data not shown). The prevention of diphenhydramine, pyrilamine and promethazine antinociception produced by anti-$Gi_1\alpha$, anti-$Gi_2\alpha$ and anti-$Gi_3\alpha$ at the highest active dose (12.5–25 μg per mouse i.c.v.), disappeared 7 days after the end of the aODN pretreatment (Fig. 4).

Anti-$Gi\alpha_1$ (12.5 μg per mouse i.c.v.), anti-$Gi\alpha_2$ (25 μg per mouse i.c.v.) and anti-$Gi\alpha_3$ (25 μg per mouse i.c.v.)

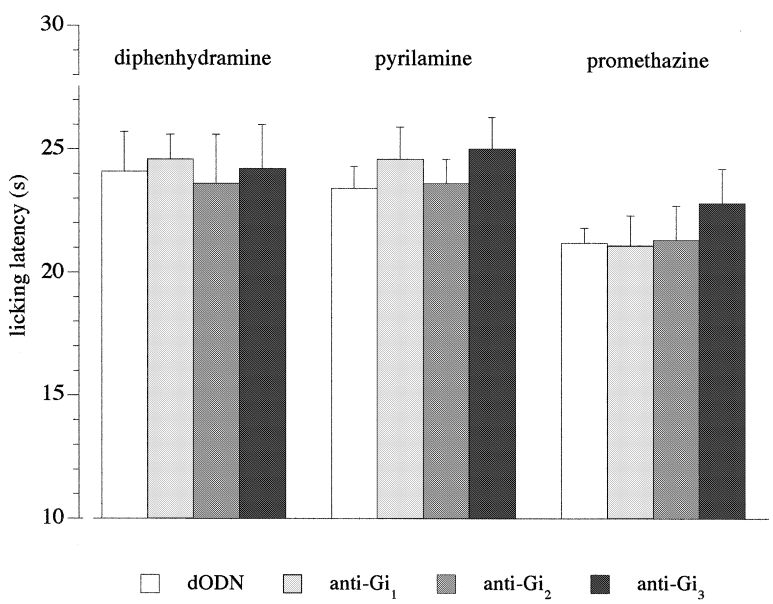

Fig. 4. Lack of effect by pretreatment with an aODN to the α subunit of Gi_1- (12.5 μg per mouse i.c.v.), Gi_2- (25 μg per mouse i.c.v.) and Gi_3- (25 μg per mouse i.c.v.) protein gene on diphenhydramine (20 mg kg^{-1} s.c.), pyrilamine (15 mg kg^{-1} s.c.) and promethazine (6 mg kg^{-1} s.c.) antinociception 7 days after the i.c.v. injection of dODN (12.5–25 μg per mouse i.c.v.) or aODN. Vertical lines represent S.E.M. Fifteen mice per group were tested.

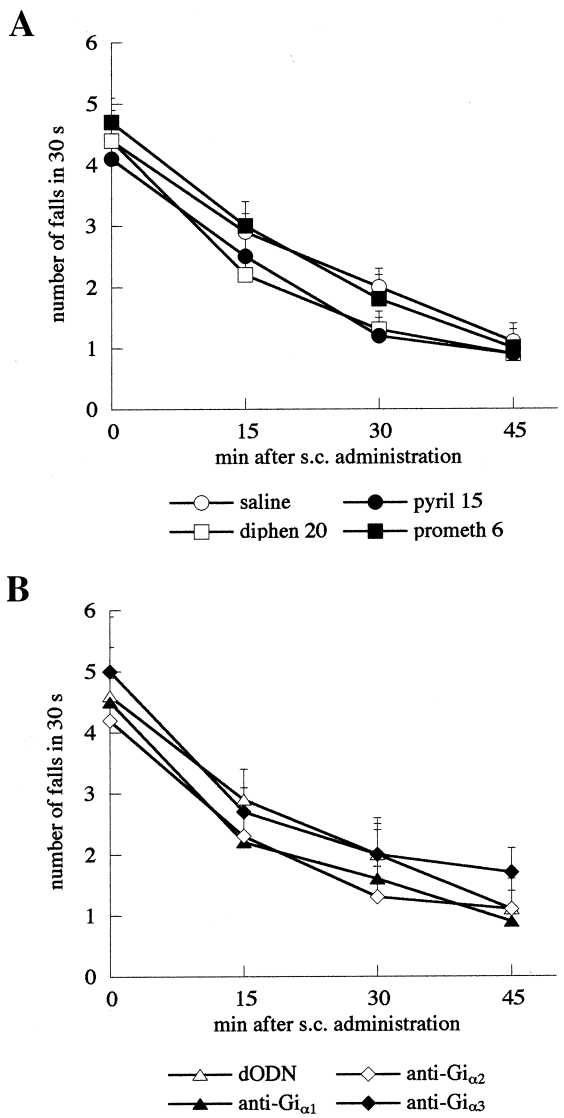

Fig. 5. (A) Effect of diphenhydramine (20 mg kg^{-1} s.c.), pyrilamine (15 mg kg^{-1} s.c.) and promethazine (6 mg kg^{-1} s.c.) on motor coordination in the mouse rota rod test. Antihistamines were administered immediately before the test. (B) Effect of pretreatment with an aODN to the α subunit of Gi_1- (12.5 µg per mouse i.c.v.), Gi_2- (25 µg per mouse i.c.v.) and Gi_3- (25 µg per mouse i.c.v.) protein gene on motor coordination in the mouse rota rod test. The test was performed 18 h after the last i.c.v. injection of dODN (25 µg per mouse i.c.v.) or aODN. Vertical lines represent S.E.M. Twelve mice per group were tested.

did not produce any modification of the pain threshold in comparison with dODN-treated mice when given alone (Figs. 1–3).

Pretreatment with dODN never modified the animals' sensitivity to the analgesic treatments in comparison with saline- or DOTAP-injected mice (i.c.v.) and with unpretreated animals (data not shown).

Effect of H_1-antihistamines on mouse behaviour

Pyrilamine, diphenhydramine and promethazine, at the doses used in the present work, elicited their antinociceptive effect without changing gross behaviour of mice.

The three H_1-antihistamines investigated did not alter the mice motor coordination as revealed by the rota rod test (Fig. 5A). The rota rod endurance time of mice treated with diphenhydramine (20 mg kg^{-1} s.c.), pyrilamine (15 mg kg^{-1} s.c.) and promethazine (6 mg kg^{-1} s.c.) was not modified in comparison with saline-treated mice (Fig. 5A).

Effect of aODN on mouse behaviour

The motor coordination of mice pretreated with aODN to $Gi_1\alpha$ (12.5 µg per mouse i.c.v.), $Gi_2\alpha$ (25 µg per mouse i.c.v.) and $Gi_3\alpha$ (25 µg per mouse i.c.v.) was evaluated by using the rota rod test. The motor coordination of aODN-treated groups was not impaired when compared with that of dODN-treated mice (Fig. 5B).

The spontaneous motility and exploratory activity of mice was not modified by administration of the above-mentioned aODNs as revealed by the hole board test in comparison with dODN-treated mice (Fig. 6). In the same experimental conditions D-amphetamine (1 mg kg^{-1} i.p.), used as the reference drug, increased both parameters evaluated (Fig. 6).

Furthermore, pyrilamine (15 mg kg^{-1} s.c.), diphenhydramine (20 mg kg^{-1} s.c.) and promethazine (6 mg kg^{-1} s.c.) did not modify the mice spontaneous motility (data not shown).

DISCUSSION

The important role played by the Gi-protein family in the mechanism of antinociceptive action of antihistamines is indicated by present results. In particular, the involvement of Gi_1, Gi_2 and Gi_3 protein subtypes has been observed. The administration of aODN against the α subunit of the above-mentioned Gi-proteins produced a dose-dependent prevention of the antinociception induced by diphenhydramine, pyrilamine and promethazine in the mouse hot-plate test.

These data also indicate that antihistamines exert their antinociceptive effect by acting centrally. The antagonism of the diphenhydramine, pyrilamine and promethazine antinociception, by i.c.v.-injected aODNs shows that the site of action of antihistamines is centrally located.

The inhibition of antihistamine-induced enhancement of the pain threshold disappeared 7 days after pretreatment with the aODNs. This return of sensitivity implies both the total reversal of aODN-induced specific inhibition of Giα expression and a lack of damage or toxicity associated with aODN treatment. Pretreatment with the anti-Giα ODNs at the highest effective doses did not modify the pain threshold, showing the absence of any hyperalgesic effect. Therefore, the prevention of antihistamine antinociception cannot be attributable to a direct effect on the pain threshold induced by the aODN. Furthermore, the dODN did not modify diphenhydramine-,

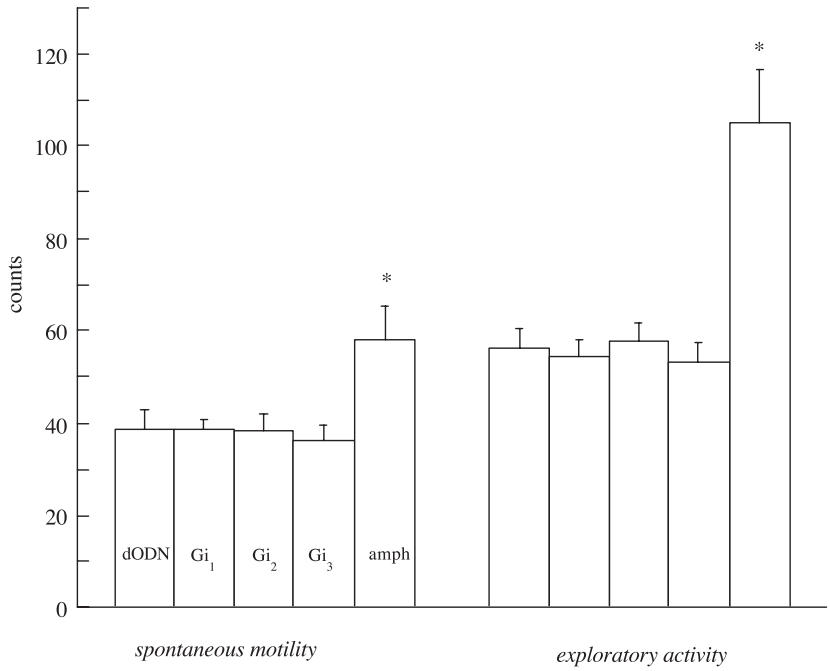

Fig. 6. Lack of effect of pretreatment with an aODN to the α subunit of Gi_1- (12.5 μg per mouse i.c.v.), Gi_2- (25 μg per mouse i.c.v.) and Gi_3- (25 μg per mouse i.c.v.) protein gene on spontaneous motility and inspection activity in the mouse hole board test. The test was performed 18 h after the last i.c.v. injection of dODN (25 μg per mouse) or aODN. Vertical lines represent S.E.M. Twelve mice per group were tested. Amphet: D-amphetamine 1 mg kg^{-1} i.p. $*P < 0.05$ in comparison with saline group.

pyrilamine- and promethazine-induced antinociception in comparison with naive or saline-i.c.v.-injected mice (data not shown). This observation ruled out the possibility that the antagonism exerted by aODNs may have resulted from a sequence-independent action on cerebral structures.

Diphenhydramine, pyrilamine and promethazine are first-generation H_1-receptor antagonists (Simons and Simons, 1994). H_1 receptors are G-protein coupled receptors that activate phospholipase C via a PTX-insensitive mechanism (Arrang, 1994). By contrast, the antagonism exerted by anti-Giα indicates that the intracellular mechanism of analgesic action of H_1-antihistamines involves the activation of PTX-sensitive G-proteins. These results confirm and extend previous data which evidenced that diphenhydramine, pyrilamine and promethazine antinociception is prevented by the i.c.v. administration of PTX (Galeotti et al., 1996; 1999). It has been reported that first-generation H_1-receptor antagonists are receptor-independent G-protein activators in HL-60 cells, basophils and mast cells and that such a mechanism of action might be responsible for the stimulatory effects of these compounds (Burde et al., 1996). We can, therefore, suppose that a direct activation of the Gi-protein-mediated transduction system might also be responsible for the increase of the pain threshold induced by antihistamines. However, first-generation H_1-receptor antagonists induce histamine release from basophils and mast cells (Mota and Da Silva, 1960; Mota, 1966; Lichtenstein and Gillespie, 1975) and increase the histamine levels in the cerebrospinal fluid (Suojaranta-Ylinen et al., 1991). Since it is well known that histamine can modulate the release of several neurotransmitters (Hill, 1990), an involvement of neurotransmitters different from histamine, activating Gi-proteins as a signal transduction mechanism, downstream from the initial receptor interaction, cannot be excluded.

Taking into account present observations together with several literature data, it appears that the Gi-protein system represents an essential intracellular step in the induction of central antinociception. An increase of the pain threshold, regardless the receptor subtype stimulated or the neurotransmitter system involved, seems to underlie the activation of Gi-proteins as a common intracellular event. Hypofunctionality of PTX-sensitive G-proteins not only induces hyperalgesia (Ohnishi et al., 1990; Galeotti et al., 1996; Womer et al., 1997), but also produces insensitivity to analgesic treatments. Inactivation of Gi-proteins prevents the analgesic activity of inhibitory neurotransmitters such as β-endorphins, GABA, cathecholamines, purines (Chung et al., 1994; Hoehn et al., 1988; Sawynok and Reid, 1988) as well as the induction of analgesia by well-known analgesic drugs such as opioids, tricyclic antidepressants, α_2-adrenoceptor agonists (Parenti et al., 1986; Sanchez-Blazquez and Garzon, 1991; Galeotti et al., 1996).

As the Gi-proteins are widely distributed in the neural areas, the function of a variety of cellular receptors is expected to be altered by the impairing effect of the antisense treatment. It has been, therefore, necessary to evaluate the possible induction of side effects by the anti-Giα treatment. The aODNs, at the highest doses used, did not modify animals' gross behaviour. Moreover, additional behavioural tests were performed to illustrate any side effects produced by the treatments that cannot be revealed by the researcher through the observation of

the animal's spontaneous behaviour. The aODN treatment did not impair motor coordination, as revealed by the progressive reduction of the number of falls in the rota rod test. Repetition of the test session every 15 min progressively decreases the number of falls since animals learn how to balance on the rotating rod. The lack of variation, or an increase in the number of falls after treatment indicates an impairment of mice motor coordination that could lead to a misinterpretation of the results obtained in the analgesic test. Mice, even if still sensitive to the thermal stimulus used in the hot-plate test, may have no reaction to pain (licking of paws) because of their impaired motility. Moreover, aODNs did not modify spontaneous motility and exploratory activity as indicated by the hole board test. We can, thus, suppose that the effects observed in the hot-plate test were not imputable to compromised behavioural paradigms.

Similarly, the administration of histamine H_1 receptor antagonists produces various inhibitory effects including sedation (Simons and Simons, 1994) whose appearance could lead to a modification of the licking latency values observed in the hot-plate test. At the doses employed, diphenhydramine, pyrilamine and promethazine exerted their antinociceptive activity without showing any alteration of the mice motor coordination as revealed by the rota rod test. Furthermore, the antihistamines did not modify the spontaneous motility and the exploratory activity as revealed by the hole board test (data not shown).

In conclusion, present results evidence the important role of Gi_1, Gi_2 and Gi_3 protein subtypes in the mechanism of analgesic action of diphenhydramine, pyrilamine and promethazine.

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