DIPHENHYDRAMINE-INDUCED AMNESIA IS MEDIATED BY Gi-PROTEIN ACTIVATION

N. GALEOTTI, A. BARTOLINI AND C. GHELARDINI*
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-Giα1, anti-Giα2, anti-Giα3, anti-Goα1, anti-Goα2) on the amnesia induced by the H1-antihistamine diphenhydramine (20 mg kg⁻¹ s.c.) was evaluated in the mouse passive avoidance test. Pretreatment with anti-Giα1 (12.5–25 μg per mouse i.c.v.) and anti-Giα2 (25 μg per mouse i.c.v.), administered 24 and 18 h before test, prevented antihistamine-induced amnesia. By contrast, pretreatment with an anti-Giα3 (25 μg per mouse i.c.v.), anti-Goα1 (25 μg per mouse i.c.v.) and anti-Goα2 (25 μg per mouse i.c.v.) did not modify the detrimental effect induced by diphenhydramine. 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 the important role played by the Giα1- and Giα2-protein pathway in the transduction mechanism involved in the impairment of memory processes produced by the H1-antihistamine diphenhydramine. © 2003 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: Gi-proteins, memory, amnesia, H1-antihistamines, diphenhydramine, passive avoidance.

The H1-receptor antagonists are amongst the most widely used medications in the world. These compounds produce the inhibition of the effects of histamine mediated by H1 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 (Rimmer and Church, 1990). In addition to these well-known peripheral effects, H1-receptor antagonists produce various central inhibitory actions (Simons and Simons, 1994). The first generation of H1 antagonists can both stimulate and depress the CNS. Stimulation occasionally is encountered in patients given therapeutic doses, but it is a striking feature of poisoning, which can result in convulsions, particularly in infants (Faingold and Berry, 1972). Central depression, on the other hand, usually accompanies therapeutic doses of the H1 antagonists, which appears to be related to occupancy of cerebral H1 receptors (Simons and Simons, 1994). Diminished alertness, slowed reaction times or somnolence are common manifestations (Simons and Simons, 1994). Several data also indicate the induction of an impairment of cognitive functions by antihistamines. The post-training i.c.v. administration of histamine has been reported to cause memory facilitation in rats which is antagonized by the simultaneous treatment with both promethazine and cimetidine (de Almeida and Izquierdo, 1986, 1988). Moreover, the administration of diphenhydramine further reduces learning ability in patients with seasonal allergic rhinitis in which learning performances are already impaired by allergy symptoms (Vuurman et al., 1996).

Even if the amnesic properties of antihistamines are documented, the post-receptorial mechanism involved in the induction of this detrimental effect on cognitive processes has not yet been established. It has been reported that first-generation H1-receptor antagonists are receptor-independent G-protein activators in HL-60 cells, basophils and mast cells (Burde et al., 1996). Recently, it has been reported that one of the central effects induced by H1-receptor antagonists, the anticonception, 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). Furthermore, the i.c.v. administration of antisense oligodeoxynucleotides directed against the α subunit of different Gi-proteins (anti-Giα1, anti-Giα2, anti-Giα3) prevented the antinociception induced by antihistamines (Galeotti et al., 2002). Since Gi-proteins represent the most widespread modulatory signalling pathway in neurones (Holz et al., 1986), we thought it worthwhile to investigate the involvement of Gi proteins into the mechanism of action of the antihistamine diphenhydramine, by means of an antisense strategy. The Gi protein family is composed by several subtypes, named Giα1, Giα2, Giα3, Goα1, and Goα2 (Simon et al., 1991). In the current study we used antisense oligonucleotides (aODN) against the α subunits of the Giα1, Giα2, Giα3, Goα1, and Goα2 proteins in order to determine the role of each subtype in the memory impairment induced by diphenhydramine in a mouse passive avoidance paradigm. In order to exclude that the effects produced by aODN treatments were due to the induction of side effects, some additional behavioural tests (rota rod, hole board) were performed.
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 acclimatization. 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 7:00 a.m. 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 anesthesia, according to the method described by Haley and McCormick (1957). Briefly, during anesthesia, 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.

Passive-avoidance test

The test was performed according to the step-through method described by Jarvik and Kopp (1967). The apparatus consisted of a two-compartment acrylic box with a lighted compartment connected to a darkened one by a guillotine door. As soon as the mouse entered the dark compartment, it received a punishing electrical shock (0.5 mA, 1 s). The latency times for entering the dark compartment were measured in the training test and after 24 h in the retention test. The maximum entry latency allowed in the training and retention sessions was, respectively, 60 and 24 h in the retention test. The maximum entry latency allowed in each session was 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 the beginning of the test. 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 (Genoa, Italy). The sequences of the 33-mer aODNs used in the present study were the following: anti-Gi, 5’-G’G’C’T GTC CTT CCA CAG TCT CTT TAT GTC AGC CCA GAG CCT CCG GAT GAC GCC G’G’A’-3’; anti-Gi, 5’-A’T’G GTC AGC CCA GAG CCT CCG GAT GAC GCC G’G’A’-3’; anti-Gi, 5’-G’G’C’C’ ATC TCG CCA TAA ACG TTT AAT CAC GCC G’G’C’-3’; anti-Go, 5’-A’G’G’ CAG CTG CAT CTT CAT AGG TG T* T’ T’ T’ -3’; anti-Go, 5’-G’A’G’ CCA CAG CTT CTG TGA AGG CA’C’ * T’ -3’. All ODNs were previously characterised by in vitro (immunoblotting) and in vivo (tail flick) experiments (Kleuss et al., 1991; Raffa et al., 1994; Sanchez-Blazquez et al., 1995; Sanchez-Blazquez and Garzon, 1998). We also confirmed the aODN effect on Gi/ou protein levels by performing immunoblotting experiments. We observed a statistically significant reduction of the expression of Gi1 (36.4±10.6), Gi2 (38.9±13.6), Gi3 (41.1±8.9), Go1 (39.8±11.1), Go2 (37.1±12.5) levels after aODN treatment (25 μg per mouse i.c.v.) in comparison with mice treated with dODN (25 μg per mouse i.c.v.). The dODN treatment did not modify the Gi/ou protein levels in comparison with saline-treated mice. At the dose of 6.5 μg per mouse i.c.v., we did not observe any significant modification of the protein levels (data not shown). A 33-mer fully dODN 5’-N’N’N NN NNN NNN NNN NNN NNN NNN N’N’N’N’ (where N is G, or C, or A, or T) and a 25-mer fully dODN 5’-N’N’N NNN NNN NNN NNN NNN NNN N’N’N’ (where N is G, or C, or A, or T) were used as a control respectively for anti-Gi and anti-Go. ODNs were vehiculated intracellularly by an artificial cationic lipid (DOTAP, Sigma, Milan, Italy) 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: diphenhydramine hydrochloride, c-aminophenamine hydrochloride (De Angelis). 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, followed by Fisher’s protected least significant difference procedure for post hoc comparison, was used to verify significance between two means. Data were analysed with the StatView software for the Macintosh (1992). P values of <0.05 were considered significant.
RESULTS

Effect of aODN against Gi\textsubscript{1} subunits on diphenhydramine amnesia

Diphenhydramine (20 mg kg\textsuperscript{-1} s.c.) induced amnesia in the mouse passive avoidance test that was prevented by pretreatment with an aODN against the \(\alpha\) subunit of the Gi\textsubscript{1} proteins. Anti-Gi\textsubscript{1} (6.25–12.5 \(\mu\)g per mouse i.c.v.) produced a dose-dependent antagonism of the detrimental effect produced by the \(H_1\)-antihistamine. The dose of 6.25 \(\mu\)g per mouse i.c.v. was completely ineffective; 12.5 \(\mu\)g per mouse i.c.v. partially prevented diphenhydramine amnesia whilst the doses of 25 \(\mu\)g per mouse i.c.v. enhanced the entrance latency in the retention session up to a value comparable to that produced by control animals (Fig. 1).

The administration of an aODN against the \(\alpha\) subunit of the Gi\textsubscript{2} proteins (6.25–25 \(\mu\)g per mouse i.c.v.), dose-dependently prevented diphenhydramine-induced amnesia. At the concentration of 25 \(\mu\)g per mouse i.c.v., a complete prevention was obtained, at the dose of 12.5 \(\mu\)g partially prevented diphenhydramine amnesia, even if the statistical significance was not reached, whereas, at 6.25 \(\mu\)g per mouse i.c.v., the aODN pretreatment was ineffective (Fig. 2).

The administration of an aODN against the \(\alpha\) subunit of the Gi\textsubscript{3} proteins (12.5–25 \(\mu\)g per mouse i.c.v.), in contrast to anti-Gi\textsubscript{1} and anti-Gi\textsubscript{2}, was unable to prevent diphenhydramine-induced amnesia (Fig. 3). At the highest dose employed, anti-Gi\textsubscript{3} did not modify the entrance latency in mice in comparison with the control groups (Fig. 3).

A time-course of recovery from antihistamine amnesia after aODN treatment was performed. The prevention of diphenhydramine-induced impairment of memory processes produced by anti-Gi\textsubscript{1} and anti-Gi\textsubscript{2} at the highest active dose (12.5–25 \(\mu\)g per mouse i.c.v.), disappeared 7 days after the end of the aODN pretreatment (data not shown).

Effect of aODN against Go\textsubscript{1} subunits on diphenhydramine amnesia

The administration of an aODN against the \(\alpha\) subunit of the Go\textsubscript{1} and Go\textsubscript{2} proteins (25 \(\mu\)g per mouse i.c.v.) was unable to prevent diphenhydramine-induced amnesia (Fig. 4). At the highest dose employed, anti-Gi\textsubscript{2} did not modify the entrance latency in mice in comparison with the control groups (Fig. 4).

Effect of diphenhydramine on mouse behaviour

Diphenhydramine, at the dose used in the present work, elicited its detrimental effect on memory functions without changing gross behaviour of mice. The \(H_1\)-antihistamine investigated did not alter the mice motor coordination, as revealed by the rota rod test, the spontaneous motility and inspection activity, as revealed by the hole board test (data not shown).

Effect of aODNs on mouse behaviour

The motor coordination of mice pretreated with aODN to Gi\textsubscript{1} (25 \(\mu\)g per mouse i.c.v.), Gi\textsubscript{2} (25 \(\mu\)g per mouse i.c.v.), Gi\textsubscript{3} (25 \(\mu\)g per mouse i.c.v.), Go\textsubscript{1} (25 \(\mu\)g per mouse i.c.v.) and Gi\textsubscript{2} (25 \(\mu\)g 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. 5).

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).

---

**Fig. 2.** Prevention by aODN against the \(\alpha\) subunit of Gi\(_2\) protein (6.25–25 \(\mu\)g per mouse i.c.v.) of diphenhydramine (20 mg kg\(^{-1}\) s.c.) amnesia in the mouse passive avoidance test. Vertical lines represent S.E.M. **\(P<0.01\)** in comparison with dODN+diphenhydramine-treated mice.

**Fig. 3.** Lack of effect by aODN against the \(\alpha\) subunit of Gi\(_3\) protein (12.5–25 \(\mu\)g per mouse i.c.v.) of diphenhydramine (20 mg kg\(^{-1}\) s.c.) amnesia in the mouse passive avoidance test. Vertical lines represent S.E.M. *\(P<0.05\), **\(P<0.01\)** in comparison with dODN+saline-treated mice.
The important role played by the Gi-protein family in the mechanism of amnesic action of diphenhydramine is indicated by present results. The inhibition of the expression of \( \text{Gi}_1 \) and \( \text{Gi}_2 \) produced a dose-dependent prevention of diphenhydramine-induced amnesia whereas the administration of aODNs against \( \text{Gi}_3, \text{Go}_1 \) and \( \text{Go}_2 \) never exerted any modification of diphenhydramine activity in the mouse passive avoidance test. These results indicate a differential involvement of the Gi protein subtypes in the mechanism of action of the investigated H\(_1\)-antihistamine. In particular, the integrity and functionality of \( \text{Gi}_1 \) and \( \text{Gi}_2 \) proteins appears essential to produce memory impairment by diphenhydramine. By contrast, the \( \text{Gi}_3, \text{Go}_1 \) and \( \text{Go}_2 \) subtypes, in these experimental conditions, appears not to be involved.

The inhibition of antihistamine-induced memory disruption disappeared 7 days after pretreatment with the aODNs. This return of sensitivity implies both the total reversal of aODN-induced specific inhibition of \( \text{Gi}_1 \) expression and a lack of damage or toxicity associated with aODN treatment. Pretreatment with the anti-\( \text{Gi}_1 \) ODNs at the highest effective doses did not modify the entrance latency, showing the absence of any effect on memory functions by these pretreatments. Therefore, the prevention of diphenhydramine amnesia cannot be attributable to a direct beneficial effect on memory processes induced by the aODNs. Furthermore, the dODN did not modify diphenhydramine-induced amnesia 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 is a first-generation H\(_1\)-receptor antagonist (Simons and Simons, 1994). \( \text{H}_1 \) receptors are G-protein coupled receptors that activate phospholipase C via a PTX-insensitive mechanism (Arrang, 1994). By contrast, the antagonism exerted by anti-\( \text{Gi}_1 \) indicates that the intracellular mechanism of analgesic action of the investigated H\(_1\)-antihistamine involves the activation of PTX-sensitive G-proteins. It has been reported that first-generation...
H<sub>1</sub>-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 detrimental effect on memory functions induced by antihistamines. The involvement of the Gi-protein system has also been observed for other central effects of antihistamines. Literature data evidenced that diphenhydramine, pyrilamine and promethazine antinociception is prevented by the i.c.v. administration of PTX, which inactivates Gi proteins (Galeotti et al., 1996, 1999) and of aODNs against the α subunit of Gi proteins (Galeotti et al., 2002). These results further support the hypothesis of an involvement of the Gi protein-mediated system in the intracellular events responsible for the induction of amnesia by diphenhydramine. However, first-generation H<sub>1</sub>-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.

In the present study we observed the lack of effect of an aODN against Gi<sub>3</sub> that implies that this subunit is not a major component of transduction mechanisms leading to amnesia. By contrast, other pharmacological activities of diphenhydramine, as analgesia, are mediated by activation of the three Gi protein subtypes (Galeotti et al., 2002). It is plausible to suppose that different-induced effects are mediated by different Gi-protein subunits and that diphenhydramine could have different intrinsic activity for each effect.

Taking into account present observations together with several literature data, it appears that the Gi-protein sys-
tem represents an essential intracellular step in the induction of amnesia. Gi proteins were shown to inhibit the adenyllyl cyclase activity with a consequent reduction of intracellular cAMP levels (Wong et al., 1992). The role of the cAMP cascade in memory processes has been recently evidenced. Genetic and pharmacological studies in mice and rats demonstrated that the cAMP responsive element binding protein is required for a variety of complex forms of memory, including spatial and social learning (Silva et al., 1998). The activation of the cAMP-dependent protein kinase induces long-term memory (Muller, 2000) and the inhibition of cAMP phosphodiesterase reverses memory deficits in the radial arm maze task (Zhang et al., 2000). Several studies have also shown that the regulation of adenyllyl cyclase activity is disrupted in Alzheimer’s disease patients. The alteration of adenyllyl cyclase activity in postmortem brain is related to an impairment of the stimulatory G-proteins (Gs) whereas the Gi-protein-mediated inhibition of the enzyme is unaltered (Schnecko et al., 1994; Fowler et al., 1995). Furthermore, lower Gsα levels and unmodified Gisα levels were observed in fibroblasts from familial Alzheimer’s disease patients (Shanahan et al., 1997). Recently, it has been reported that amyloid β-peptides cause toxicity through activation of Gi proteins (Soomets et al., 1999; Rymer and Good, 2001). We can hypothesize that an intact Gi-protein functionality is essential for the induction of amnesia. Our results on diphenhydramine amnesia confirm the hypothesis of a Gi-protein involvement in the induction of memory impairment. By considering that high cAMP levels are required for mnemonic integrity, we can also suppose that diphenhydramine induces amnesia through a reduction of intracellular cAMP levels via the activation of Gi1 and Gi2 proteins.

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 anti-sense treatment. It has been, therefore, necessary to evaluate the possible induction of side effects by the anti-Gi 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 behavioural test. 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 passive avoidance test were not imputable to compromised behavioural paradigms.

Similarly, the administration of histamine H1 receptor antagonists produces various inhibitory effects including sedation (Simons and Simons, 1994) whose appearance could lead to a modification of the entrance latency values observed in the passive avoidance test. At the dose employed, diphenhydramine exerted its amnesic activity without showing any alteration of the mice motor coordination, spontaneous motility and exploratory activity.

In conclusion, our results evidence that knockdown of Gi1α and Gi2α, but not by Gi3α, Goα1, and Goα4, reversed the diphenhydramine-induced amnesia, indicating the involvement of this transduction system in the induction of a detrimental effect on memory functions by H1-antihistamines.

Acknowledgements—This work was supported by grants from MURST.

REFERENCES


Mota I, Da Silva WD (1960) The anti-anaphylactic and histamine-

(Accepted 4 August 2003)