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Role of muscarinic receptor subtypes in central antinociception

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1 The ability to modify the pain threshold by the two M_1-muscarinic agonists: McN-A-343 and AF-102B and by the specific M_2-agonist arecaidine was examined in mice and rats by using three different noxious stimuli: chemical (writhing test), thermic (hot-plate test) and mechanical (paw pressure test).

2 In the mouse hot-plate test McN-A-343 (20–50 μg per mouse i.c.v.) and AF-102B (1–10 mg kg^{-1} i.p.) produced significant antinociception which was prevented by atropine (1 μg per mouse i.c.v.) and by the two selective M_1 antagonists: pirenzepine (0.01 μg per mouse i.c.v.) and dicyclomine (0.08 μg per mouse i.c.v. or 10 mg kg^{-1} i.p.) but not by the specific M_2-antagonist AFDX-116 (0.1 μg per mouse i.c.v.), naloxone (1 mg kg^{-1} i.p.) or by the acetylcholine (ACh) depletor hemicholinium-3 (HC-3) (1 μg per mouse i.c.v.). McN-A-343 and AF-102B were able to increase the pain threshold also in the mouse acetic acid writhing test and in rat paw pressure test. These antinociceptive effects were completely prevented by dicyclomine (0.08 μg per mouse i.c.v. or 10 mg kg^{-1} i.p.) but not by AFDX-116 (0.1 μg per mouse or rat i.c.v.).

3 In contrast with the M_1-agonists, the M_2-agonist arecaidine (0.1–2 μg per mouse or rat i.c.v.) did not induce antinociception in all three analgesic tests. However, arecaidine, at the same i.c.v. doses, was able to reduce the pain threshold in the hot-plate and paw pressure tests.

4 The site of muscarinic control of the pain threshold is localized in the CNS since drugs which do not cross the blood-brain barrier such as McN-A-343, pirenzepine and arecaidine exerted their effects only if injected i.c.v.

5 On the basis of the above findings and existing literature we suggest that the postsynaptic muscarinic receptors involved in antinociception belong to the M_1 subtype. Nevertheless, presynaptic autoreceptors (M_2 subtype) may play a role in pain regulation since they are involved in modulation of endogenous ACh release.

Keywords: Analgesia; antinociception; pain; muscarinic receptors; muscarinic agonists; muscarinic antagonists; acetylcholine

Introduction

Intracerebroventricularly (i.c.v.) administered acetylcholine (ACh) (Pedigo *et al.*, 1975) and both direct and indirect muscarinic agonists such as oxotremorine (George *et al.*, 1962; Bartolini *et al.*, 1980; 1987a) and its precursor tremorine (Lenke, 1958), arecoline (Herz, 1962), pilocarpine (Hendershot & Forsaith, 1959; Chernov *et al.*, 1967), physostigmine (Harris *et al.*, 1969; Ireson, 1970) and diisopropyl phosphorofluoridate (DFP) (Lentz *et al.*, 1969) induce antinociception in the laboratory animal. Since such antinociception is completely antagonized by atropine (Bartolini *et al.*, 1987a), a muscarinic receptor should be involved. The postsynaptic muscarinic receptor involved in antinociception should be of the M_1-subtype, since recently Malmberg-Aiello *et al.* (1988) and Ghelardini *et al.* (1990) have demonstrated that pirenzepine, a selective M_1 antagonist (Hammer *et al.*, 1980), is also able to block oxotremorine antinociception.

We thought it important to verify whether the two selective M_1-agonists: McN-A-343 (Hammer & Giachetti, 1982; Lambrecht & Mutschler, 1985; Lambrecht *et al.*, 1986) and AF-102B (Fisher *et al.*, 1986; Ono *et al.*, 1988; Fisher *et al.*, 1989; Nakahara *et al.*, 1990) and the M_2-agonist arecaidine (Mutschler & Hultzsch, 1973; Mutschler & Lambrecht, 1984; Barlow & Weston-Smith, 1985) were able to modify the pain threshold, since muscarinic antinociception was thus far obtained only by employing agonists devoid of selectivity for various muscarinic receptor subtypes.

Moreover, in order to strengthen the previous results concerning the antagonism of muscarinic antinociception by pirenzepine we investigated the antagonistic effect on antinociception of dicyclomine, another well known M_1-antagonist (Nilvebrant & Sparf, 1986). For comparison with the effect of the M_1-antagonists, the action of the selective M_2-antagonist AFDX-116 (Giachetti *et al.*, 1986a,b) was also tested.

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Methods

Hot-plate test

Male Swiss albino mice (25–30 g) from the Morini breeding farm were used. The mice were placed inside a stainless steel container thermostatically set at 52.5 ± 0.1°C in a precision water-bath from KW Mechanical Workshops, Siena, Italy. The reaction times (s) were measured with a stop-watch before, and 15, 30, 45 and 60 min after treatment. The end-point used was the licking of the fore or hind paws. Those mice which scored below 12 or over 18 s in the pretest were rejected. An arbitrary cut-off time of 45 s was adopted.

Writhing test

Male Swiss albino mice (25–30 g) were injected i.p. with a 0.5% aqueous solution of acetic acid (10 ml kg^{-1}). The number of stretching movements was counted for 10 min, starting 5 min after acetic acid injection.

Paw pressure (Randall Selitto test)

The nociceptive threshold was determined with an analgesimeter (Ugo Basile, Varese, Italy) in rats. The instrument exerts a force which is applied at a constant rate (32 g per second) with a cone-shaped pusher on the upper surface of the rat hind paw. The force is continuously monitored by a pointer moving along a linear scale. The pain threshold is given by the force which induces the first struggling from the rat. Those pretested rats which scored below 30 g or over 90 g were rejected. An arbitrary cut-off value of 200 g was adopted.

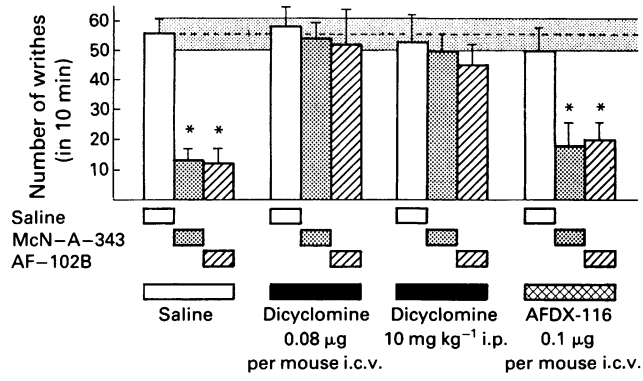

Figure 1 Effect of dicyclomine (0.08 μg per mouse – 10 mg kg^{-1} i.p.) and AFDX-116 (0.1 μg per mouse) pretreatments on antinociception induced by McN-A-343 (30 μg per mouse i.c.v.) and AF-102B (5 mg kg^{-1} i.p.) in the mouse writhing test. Dicyclomine and AFDX-116 were injected respectively 15 and 5 min before the administration of cholinomimetics. The nociceptive responses were recorded 15 min after administration of McN-A-343 and 30 min after AF-102B. Vertical lines show s.e.mean. * $P < 0.01$ in comparison with saline controls. Each column represents the mean of at least ten mice.

Drugs and reagents

The following drugs were used: atropine sulphate, hemicolinium-3 hydrobromide (HC-3) and naloxone hydrochloride (Sigma), dicyclomine hydrochloride (Lepetit), pirenzepine hydrochloride (Istituto Sieroterapico Milanese), AFDX-116 and McN-A-343 (De Angeli), AF-102B (Inst. for Neurobiol. Res. Bruxelles, Belgium), arecaidine propargyl ester (Dept. of Pharmacol. Univ. of Frankfurt, Germany). Other chemicals were of the highest quality commercially available. All drugs were dissolved in a physiological saline solution. Drug concentrations were prepared in such a way that the necessary dose could be administered in a volume of 10 ml kg^{-1} by both s.c. and i.p. injection. The doses given in the text referring to atropine, HC-3, dicyclomine and pirenzepine are expressed as salts. I.c.v. administration was performed during brief ether anaesthesia according to the method described by Haley & McCormick (1957), by injecting mice and rats with the necessary dose dissolved in 5 and 10 μl of saline solution, respectively. In some experiments, mice but not rats received two i.c.v. injections performed at 5 and 15 min intervals. In such cases ether anaesthesia was repeated two times and each cerebral ventricle received only one injection.

Doses of muscarinic antagonist drugs were selected, by preliminary experiments, as the lowest ones able to prevent agonist effects.

Statistical analysis

Results are given as the mean ± s.e.mean. Dunnett's two-tailed test was used to verify the significance of differences between the means, and these were considered significant when P values were less than 0.05.

Results

Pain threshold effect of M_1- and M_2-selective agonists

McN-A-343 and AF-102B induced a significant increase in the pain threshold in both mice and rats in all three tests (Tables 1 and 2, Figures 1, 2 and 3). Antinociception from i.c.v. McN-A-343 reached a maximum after 15 min while that from i.p. AF-102B reached a maximum after 30 min. Both antinociceptive effects persisted unchanged for up to 45 min and then diminished 60 min after administration (Tables 1 and 2; Figures 2 and 3).

McN-A-343 exerted its antinociceptive activity by i.c.v. administration starting from 20 μg per mouse. Antinociception was dose-dependent up to a maximum of 50 μg per mouse, after which cholinergic symptoms (tremors and general prostration) invalidated a correct evaluation of the pain threshold. McN-A-343 (0.5 mg kg^{-1} s.c.), if injected parenterally, induced cholinergic symptoms approximately of the same intensity as those produced by oxotremorine (40 μg kg^{-1} s.c.). However, while parenterally administered oxotremorine induced antinociception (Ghelardini *et al.*, 1990), parenterally administered McN-A-343 did not.

AF-102B was able to induce antinociception when injected either intraperitoneally starting at 1 mg kg^{-1} i.p. or i.c.v. (1–5 μg per mouse) (data not shown). Weak cholinergic side-effects became visible after 10 mg kg^{-1} i.p. At higher i.p. doses side-effects did not permit testing.

In contrast to the antinociception induced by M_1-agonists, the selective M_2-agonist arecaidine produced a significant hyperalgesic action in the mouse hot-plate test (Table 3a) and rat paw pressure test (Table 3b). Arecaidine, although injected at the same i.c.v. doses used in the hot-plate and paw-pressure

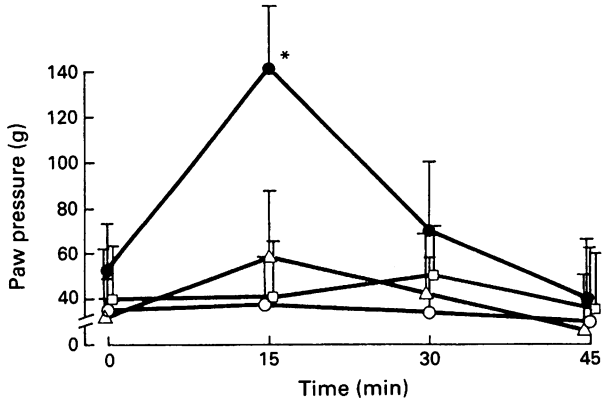

Figure 2 Time course of pain threshold after McN-A-343 in the rat paw pressure test. Antagonism by dicyclomine of antinociceptive effect induced by McN-A-343. Dicyclomine was injected 30 min before test. Vertical lines show s.e.mean. * $P < 0.01$ in comparison with saline controls. Each point is the mean of at least six rats. Saline (□); McN-A-343 30 μg per rat, i.c.v. (●); dicyclomine 10 mg kg^{-1}, i.p. (○); dicyclomine + McN-A-343 (△).

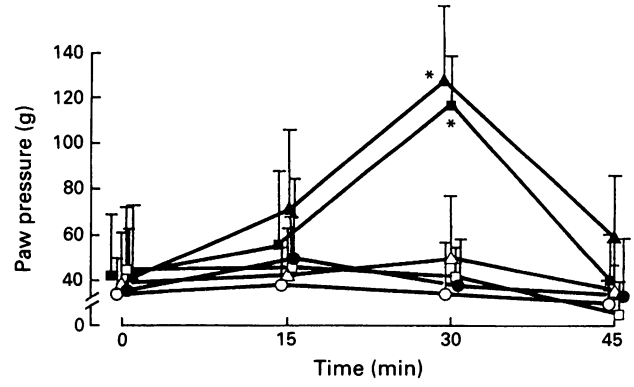

Figure 3 Time course of pain threshold after AF-102B in rat paw pressure test. Antagonism by dicyclomine occurred but not by AFDX-116 on AF-102B antinociception. Dicyclomine and AFDX-116 were injected respectively 30 min and 20 min before test. Vertical lines show s.e.mean. * $P < 0.01$ in comparison with saline controls. Each point represents the mean of at least six rats. Saline (□); AF-102B 5 mg kg^{-1}, i.p. (▲); dicyclomine 10 mg kg^{-1}, i.p. (○); dicyclomine + AF-102B (△); AFDX-116 0.1 μg per rat, i.c.v. (●); AFDX-116 + AF-102B (■).

Table 1 Effect of atropine, pirenzepine, dicyclomine, HC-3, naloxone and AFDX-116 on antinociception caused by McN-A-343

			Hot-plate *Licking latency in mice* (s)				
	Treatment	*μg per*	*Before*	*After treatment*			
Pretreatment	(i.c.v.)	*mouse*	*treatment*	*15 min*	*30 min*	*45 min*	*60 min*
Saline	Saline		15.6 ± 0.8	16.2 ± 1.0	15.8 ± 0.9	15.3 ± 1.1	16.1 ± 1.3
(i.p., 10 ml kg^{-1}	McN-A-343	10	15.0 ± 0.7	14.6 ± 1.3	15.7 ± 1.2	13.6 ± 1.1	14.9 ± 1.7
or i.c.v.,	McN-A-343	20	14.5 ± 0.5	21.1 ± 2.2*	18.2 ± 2.1	15.1 ± 1.9	16.3 ± 1.5
5 μl per mouse)	(x) McN-A-343	30	15.1 ± 0.4	27.6 ± 1.1**	26.6 ± 1.8**	22.6 ± 1.5*	17.5 ± 1.6
	McN-A-343	50	15.3 ± 0.5	42.1 ± 4.3**	40.5 ± 3.7**	35.5 ± 2.5**	22.8 ± 2.1*
Atropine	Saline		14.7 ± 0.8	13.9 ± 1.7	16.1 ± 2.2	15.7 ± 2.1	16.1 ± 2.3
(i.c.v.	McN-A-343	30	15.5 ± 0.7	17.3 ± 1.6	15.6 ± 1.9	14.2 ± 1.7	15.4 ± 1.9
1 μg per mouse)							
Pirenzepine	Saline		14.7 ± 1.3	14.1 ± 1.6	15.7 ± 1.3	16.1 ± 1.8	15.9 ± 1.7
(i.c.v.,	McN-A-343	30	13.7 ± 1.0	16.8 ± 1.7	17.1 ± 1.6	15.8 ± 1.3	14.0 ± 1.0
0.01 μg per mouse)							
Dicyclomine	Saline		14.6 ± 1.1	13.6 ± 1.8	15.9 ± 2.1	16.8 ± 2.0	14.8 ± 1.3
(i.c.v.,	McN-A-343	30	15.7 ± 0.8	17.7 ± 1.6	16.8 ± 1.9	15.4 ± 1.8	13.9 ± 2.1
0.08 μg per mouse)							
HC-3	Saline		15.3 ± 0.8	16.2 ± 1.5	16.0 ± 2.2	14.4 ± 2.3	13.6 ± 2.1
(i.c.v.,	McN-A-343	30	15.5 ± 1.0	26.9 ± 2.9**	24.6 ± 1.3**	22.5 ± 2.4**	17.1 ± 1.8
1 μg per mouse)							
Naloxone	Saline		14.9 ± 1.3	14.4 ± 1.6	15.6 ± 2.1	15.5 ± 1.8	16.3 ± 1.7
(i.p.,	McN-A-343	30	14.5 ± 1.2	25.5 ± 2.0**	23.8 ± 1.8**	25.5 ± 2.7**	14.4 ± 1.8
1 mg kg^{-1})							
AFDX-116	Saline		14.9 ± 1.0	16.1 ± 2.2	16.3 ± 1.7	14.7 ± 1.9	13.0 ± 1.2
(i.c.v.,	McN-A-343	30	15.1 ± 1.2	25.9 ± 2.9**	24.1 ± 2.3*	20.1 ± 2.1*	14.8 ± 2.5
0.1 μg per mouse)							

The number of mice ranged from 8–20 with the exception of (x) where $n = 50$.
* $P < 0.05$, ** $P < 0.01$ in comparison with saline-saline.

tests, did not modify either the number of writhes induced by i.p. acetic acid in mice (Figure 4), or the hyperalgesia induced by naloxone (1 mg kg^{-1} i.p.) in the same test (Figure 4).

Arecaidine showed its hyperalgesic effect from 15 to 30 min after i.c.v. administration in the hot-plate test and from 30 to 60 min in the rat paw pressure test (Table 3a,b). The decrease in the pain threshold was dose-dependent starting in mice at the dose of 0.1 μg and in rats at the dose of 0.5 μg. Higher doses than 2 μg per mouse or rat induced a general state of prostration. No cholinergic symptoms were present in the range of i.c.v. doses used.

Arecaidine, i.p. was also able to induce an equipotent hyperalgesic effect either in rats (10 mg kg^{-1}) or in mice (20 mg kg^{-1}) (data not shown). Hyperalgesia began after 15 min and persisted up to 30 min. By using these doses no cholinergic symptoms were observed. However, higher doses

Table 2 Effect of atropine, pirenzepine, dicyclomine, HC-3, naloxone, AFDX-116 on antinociception caused by AF 102B

			Hot-plate *Licking latency in mice* (s)				
	Treatment		*Before*	*After treatment*			
Pretreatment	(i.p.)	mg kg^{-1}	*treatment*	*15 min*	*30 min*	*45 min*	*60 min*
Saline	Saline		16.4 ± 0.9	15.1 ± 1.3	16.0 ± 1.0	14.5 ± 1.0	15.3 ± 0.7
(i.p.,	AF 102B	0.1	14.9 ± 1.1	14.1 ± 1.6	15.6 ± 1.9	16.3 ± 1.2	14.8 ± 1.9
10 ml kg^{-1})	AF 102B	1	15.1 ± 0.9	20.7 ± 1.3*	21.5 ± 2.8*	22.3 ± 2.4*	16.9 ± 1.8
	(x) AF 102B	5	15.6 ± 0.7	21.3 ± 1.1*	27.1 ± 1.7**	26.8 ± 1.9**	18.2 ± 1.3
	AF 102B	10	15.8 ± 0.8	25.8 ± 2.3**	30.1 ± 2.0**	25.9 ± 2.1**	19.0 ± 1.7
Atropine	Saline		15.6 ± 0.8	14.2 ± 1.4	16.3 ± 1.8	15.1 ± 1.0	14.3 ± 1.3
(i.c.v.,	AF-102 B	5	14.2 ± 0.7	15.4 ± 1.8	15.3 ± 1.9	14.6 ± 2.1	15.1 ± 1.6
1 μg per mouse)							
Firenzepine	Saline		15.6 ± 0.9	16.3 ± 1.0	13.6 ± 1.9	16.1 ± 1.5	13.8 ± 1.7
(i.c.v.,	AF 102B	5	15.4 ± 0.6	15.5 ± 1.6	17.3 ± 1.6	16.9 ± 1.4	15.7 ± 1.7
0.01 μg per mouse)							
Dicyclomine	Saline		15.6 ± 1.0	14.2 ± 1.6	15.8 ± 1.8	14.8 ± 1.3	15.2 ± 1.6
(i.c.v.,	AF 102B	5	14.3 ± 0.9	17.3 ± 2.1	16.2 ± 1.4	16.9 ± 1.7	16.1 ± 1.7
0.08 μg per mouse)							
HC-3	Saline		14.8 ± 0.9	13.8 ± 1.5	14.4 ± 1.6	15.5 ± 1.3	16.0 ± 1.1
(i.c.v.,	AF 102B	5	15.3 ± 1.0	19.0 ± 0.7*	26.4 ± 2.0**	25.8 ± 1.2**	17.4 ± 1.7
1 μg per mouse)							
Naloxone	Saline		14.1 ± 1.1	15.3 ± 1.0	16.9 ± 1.3	14.4 ± 1.2	15.8 ± 1.0
(i.p.,	AF 102B	5	15.1 ± 0.9	19.0 ± 2.6	25.8 ± 1.9**	27.0 ± 2.1**	18.1 ± 2.0
1 mg kg^{-1})							
AFDX-116	Saline		14.9 ± 1.0	16.1 ± 2.2	16.3 ± 1.7	14.7 ± 1.9	15.0 ± 1.2
(i.c.v.,	AF 102B	5	15.2 ± 1.1	20.9 ± 2.3*	26.6 ± 1.8**	25.3 ± 1.7**	17.3 ± 2.1
0.1 μg per mouse)							

The number of mice ranged from 8–20 with the exception of (x) wehre $n = 45$.
* $P < 0.05$, ** $P < 0.01$ in comparison with saline-saline.

Table 3 Effect of HC-3 (a and b) and AFDX-116 (a) on hyperalgesia caused by arecaidine in (a) mouse hot plate test and (b) rat paw pressure test

a

			Hot-plate Licking latency in mice (s)			
Pretreatment (i.c.v.)	*Treatment* (i.c.v.)	*μg per mouse*	*Before treatment*	*After treatment 15 min*	*30 min*	*45 min*
Saline	(x) Saline		14.6 ± 0.7	14.8 ± 1.1	14.9 ± 1.2	14.7 ± 0.9
5 μl	Arecaidine	0.01	15.0 ± 0.9	13.8 ± 1.3	14.8 ± 1.6	15.6 ± 1.6
	Arecaidine	0.1	14.7 ± 0.9	10.2 ± 1.1*	11.3 ± 1.4*	13.9 ± 0.8
	Arecaidine	1	14.7 ± 1.3	10.6 ± 1.0*	10.5 ± 0.9*	13.8 ± 1.3
	(x') Arecaidine	2	14.9 ± 0.9	11.3 ± 1.1*	12.0 ± 0.7*	14.7 ± 1.1
HC-3	Saline		15.1 ± 1.0	15.8 ± 1.6	16.3 ± 1.9	17.1 ± 2.5
1 μg per mouse	Arecaidine	0.1	14.5 ± 0.6	13.6 ± 1.5	14.5 ± 1.6	15.9 ± 1.3
AFDX-116	Saline		14.9 ± 1.1	15.5 ± 1.6	16.4 ± 2.1	15.8 ± 1.7
0.1 μg per mouse	Arecaidine	0.1	15.6 ± 1.3	16.1 ± 1.8	14.0 ± 1.6	13.9 ± 1.9

b

			Paw-pressure in rats (g)			
	Treatment (i.c.v.)	*μg per mouse*	*Before treatment*	*After treatment 30 min*	*45 min*	*60 min*
Saline	Saline		70 ± 4.0	66 ± 6.0	74 ± 4.0	72 ± 4.0
5–10 μl	Arecaidine	0.1	70 ± 4.0	56 ± 8.0	60 ± 10	44 ± 6.0
	Arecaidine	0.5	70 ± 6.0	32 ± 2.0*	32 ± 2.0*	42 ± 2.0*
	Arecaidine	1	72 ± 6.0	38 ± 2.0*	32 ± 4.0*	32 ± 2.0*
	Arecaidine	2	76 ± 4.0	40 ± 6.0*	36 ± 4.0*	32 ± 2.0*
HC-3	Saline		70 ± 4.0	68 ± 4.0	70 ± 4.0	66 ± 6.0
1 μg per rat	Arecaidine	1	70 ± 4.0	68 ± 4.0	62 ± 6.0	54 ± 2.0

The number of animals ranged from 5–18 with the exception of (x) where $n = 35$ and (x') where $n = 25$.
* $P < 0.01$ in comparison with saline-saline.

(50 mg kg^{-1}, i.p.) induced hyperalgesia together with sialorrhea and lacrimation.

Antagonism of antinociception by muscarinic blockers

Tables 1 and 2 show that atropine (1 μg per mouse i.c.v.) administered 15 min before both McN-A-343 and AF-102B, antagonized the antinociception induced in mice by the two drugs. Similarly, Tables 1 and 2 show that i.c.v. pirenzepine (0.01 μg per mouse) and i.c.v. dicyclomine (0.08 μg per mouse) administered respectively 5 and 15 min before both McN-A-343 and AF-102B, were able to abolish their antinociceptive effect in the hot-plate and writhing tests. Moreover, dicyclomine administered i.p. (10 mg kg^{-1}) also antagonized the antinociception induced by the M_1-agonist in the rat paw pressure test (Figures 2 and 3).

Neither naloxone (1 mg kg^{-1} i.p.), nor HC-3 (1 μg per mouse i.c.v.), administered 15 min and 5 h respectively before treatment, modified antinociception induced by McN-A-343 or AF-102B (Tables 1 and 2). However, HC-3 pretreatment prevented arecaidine hyperalgesia (Table 3a and b).

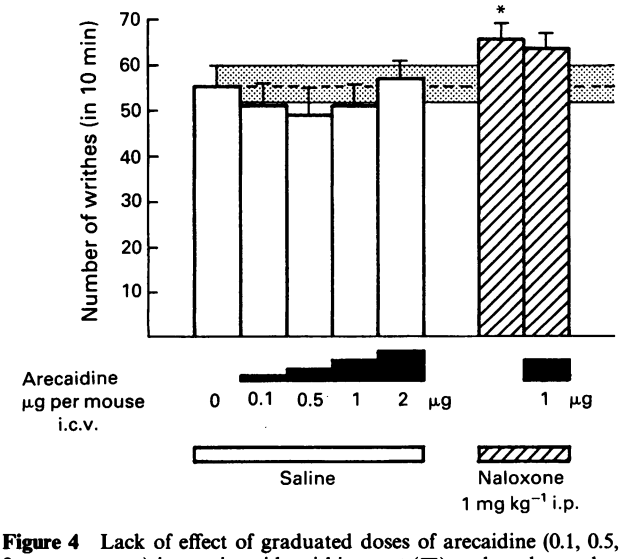

Figure 4 Lack of effect of graduated doses of arecaidine (0.1, 0.5, 1, 2 μg per mouse) in acetic acid writhing test (□) and on hyperalgesia induced by naloxone (1 mg kg^{-1} i.p.) (▨) in the same test. Arecaidine was injected 30 min before test and naloxone 5 min before arecaidine. Vertical lines show s.e.mean. * $P < 0.01$ in comparison with saline controls. Each column is the mean of at least 10 mice.

Lack of antagonism by the M_2-muscarinic blocker AFDX-116

Tables 1 and 2 and Figures 1 and 3 show that AFDX-116 did not reduce the antinociceptive activity of McN-A-343 and AF-102B. AFDX-116 was injected i.c.v. 5 min before the agonists with a dose of 0.1 μg which was the maximum usable without interfering with normal behaviour. However, at the same dose, AFDX-116 abolished arecaidine induced hyperalgesia (Table 3a).

Discussion

Our results, obtained with three different noxious stimuli (chemical, thermic and mechanical) clearly show that both the specific M_1-muscarinic agonists McN-A-343 and AF-102B are able to induce antinociception in mice and rats. Conversely, the specific M_2-agonist arecaidine did not increase the pain threshold, which was decreased in the mouse hot-plate and rat paw pressure tests.

Antinociception induced by McN-A-343 and AF-102B was antagonized by the two selective M_1-antagonists pirenzepine and dicyclomine but not by the selective M_2-antagonist AFDX-116. Therefore, it appears reasonable to suggest that only M_1-receptors are responsible for postsynaptic muscarinic antinociception. As a consequence, antinociception induced by non-specific cholinomimetics such as oxotremorine, arecoline, pilocarpine, physostigmine and DFP (see Introduction), which are able to stimulate simultaneously a variety of muscarinic receptor subtypes, appear to be due to M_1-receptor activation. Similarly, antagonism of antinociception induced by non-specific muscarinic blockers such as atropine (Bartolini *et al.*,

1987a; Ghelardini *et al.*, 1990) was due to M_1 antagonism only.

The antinociceptive effects of these drugs take place in the CNS since both agonists (McN-A-343) and antagonists (pirenzepine) which are unable to cross the blood-brain barrier exert their effects only when injected i.c.v.

The very low i.c.v. doses of pirenzepine and dicyclomine which are able to antagonize antinociception, doses respectively 100 and 12.5 times lower than that of atropine (Tables 1 and 2), show that the antagonism obtained at such low doses is selective for M_1-receptors. On the other hand, it is very difficult to extrapolate literature data concerning concentrations used in *in vitro* binding experiments, to doses employed *in vivo*. Giraldo *et al.* (1988) showed that concentrations of pirenzepine 72 times higher are necessary to displace [^{3}H]-pirenzepine from (M_2) atrial receptors compared to forebrain ($M_1 + M_2$) receptors. These authors stated that to displace ligands from M_2 (cardiac)-receptors pirenzepine concentrations above 1×10^{-6} M are necessary while to displace ligands from M_1-receptors, concentrations of pirenzepine higher than 1.4×10^{-8} M are sufficient. It is interesting to note that, the calculated concentration of the drug present in the cerebral tissue after i.c.v. injection of 10 ng (the dose necessary to antagonize muscarinic antinociception) should be about 8×10^{-8} M, assuming a uniform distribution of pirenzepine in the brain. The pirenzepine doses used therefore seem to be in the range of M_1-selectivity. Moreover the specificity of pirenzepine and dicyclomine against muscarinic receptors is further validated by the findings that these drugs, injected at the same doses needed to block antinociception caused by muscarinic agonists, are not able to modify analgesia induced by morphine and by the γ-aminobutyric acid$_B$-agonist baclofen (Ghelardini *et al.*, 1990).

AFDX-116 did not modify antinociception induced by M_1-agonists. This lack of effect might be caused by a dose of AFDX-116 inadequate to block M_2-receptors effectively. However, this possibility should be rejected since AFDX-116, at the same i.c.v. doses, was able to prevent the hyperalgesia induced by the M_2-agonist arecaidine.

On the bases of the above results (no antinociception by arecaidine and lack of antagonism by AFDX-116) an M_2 receptor involvement in postsynaptic antinociception should be ruled out. Although postsynaptic M_2-receptors are not implicated in muscarinic analgesia, presynaptic M_2 receptors play an important role in pain regulation.

There is much evidence from previous studies (Potter *et al.*, 1984; Raiteri *et al.*, 1984; Mash *et al.*, 1985; Mash & Potter, 1986; Spencer *et al.*, 1986; Pohorecki *et al.*, 1988) showing that in the CNS cholinergic inhibitory autoreceptors are of M_2-subtype. We have recently demonstrated (Bartolini *et al.*, 1987a,b; 1989; Malmberg-Aiello *et al.*, 1988; Gualtieri *et al.*, 1989; Ghelardini *et al.*, 1990) that it is possible to induce antinociception by increasing central ACh release by blocking only the presynaptic muscarinic autoreceptors.

In fact, autoreceptors could regulate pain by modulating ACh release via a negative feedback mechanism. In agreement with this hypothesis, the M_2-agonist arecaidine induced hyperalgesia which was prevented following ACh depletion with HC-3. Bartolini *et al.* (1989), Gualtieri *et al.* (1989), Ghelardini *et al.* (1990) and Iwamoto (1989) have shown that the two M_2-antagonists AFDX-116 (2–10 ng per mouse i.c.v.) and methoctramine (starting at 5 ng per mouse i.c.v.) are both able to induce antinociception in rodents.

AFDX-116 and methoctramine analgesias are prevented not only by M_1-antagonists but also by the two ACh depletors: HC-3 (Bartolini *et al.*, 1989; Gualtieri *et al.*, 1989) and vesamicol (Iwamoto, 1989). These findings suggest that both the decrease and increase in pain threshold induced by M_2 agonists and antagonists derives from decreased and increased ACh release respectively which, in turn, modulate pain via M_1-muscarinic receptors.

The observation that McN-A-343 and AF-102B preserve their antinociceptive effect even after depletion of cerebral ACh (mice pretreated with HC-3) further supports the involvement of M_1-postsynaptic receptors in analgesia.

Hyperalgesia induced by arecaidine in the hot-plate and paw pressure tests was not confirmed in the acetic acid writhing test. Since the lack of hyperalgesic effect of arecaidine in the writhing test was not removed even with naloxone pretreatment of mice, we can rule out the possibility that a simultaneous activation of the enkephalinergic system by chemical peritonitis masks arecaidine hyperalgesia.

Therefore, although a presynaptic autoreceptor hypothesis for arecaidine hyperalgesia appears to be attractive, further research is necessary in order to understand not only why arecaidine does not induce hyperalgesia in the writhing test but also why i.c.v. pirenzepine, dicyclomine and i.c.v. HC-3 do not decrease pain threshold in all three analgesic tests.

In conclusion, although M_2 presynaptic muscarinic receptors seem to play a role in cholinergic antinociception, our results have shown that the postsynaptic muscarinic receptors involved in antinociception belong to the M_1 subtype.

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