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Selective antagonists provide evidence that M_1 muscarinic receptors may mediate carbachol-induced drinking in the rat

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The present study served to investigate the ability of seven selective muscarinic antagonists to inhibit carbachol-induced drinking in the rat. The muscarinic antagonists were given by intracerebroventricular (i.c.v.) injection 1 min before the i.c.v. injection of carbachol (1 μ g/rat). The M_2 antagonist, methoctramine, was inactive up to 80.3 nmol/rat. The M_3 antagonist, p-fluoro-hexahydro-sila-difenidol, elicited a modest (42%) but statistically significant inhibition of drinking only at 80 nmol/rat. On the other hand, the selective M_1 antagonists, (R)-trihexyphenidyl, o-methoxy-sila-hexocyclium and pirenzepine, produced a marked and dose-dependent inhibition of carbachol-induced drinking, their ID_{50} values being 0.51, 7.36 and 9.31 nmol/rat. Also the M_1/M_3 antagonists, 4-diphenylacetoxy-N-methylpiperidine methiodide and hexahydro-sila-difenidol, were potent inhibitors of carbachol-induced drinking, their ID_{50} values (0.28 and 11.09 nmol/rat) being related to their pA_2 values for M_1 receptors in rabbit vas deferens. These data suggest that carbachol-induced drinking may be mediated by activation of muscarinic M_1 receptors.

Carbachol-induced drinking; Muscarinic receptor antagonists; Muscarinic receptor subtypes; Muscarinic M_1 receptors; Muscarinic M_2 receptors; Muscarinic M_3 receptors

1. Introduction

The first indication of muscarinic receptor heterogeneity was derived from studies on agonist binding (Birdsall et al., 1980). However, agonist binding curves are difficult to interpret in terms of receptor subtypes, owing to the complexity of agonist-receptor interaction, involving both different affinity states and coupling proteins. The discovery of antimuscarinic compounds which are selective for certain tissues or organs provided a more reliable approach to the subclassification of

muscarinic receptors. On this basis, these receptors have been classified into at least three subtypes: M_1 (Hammer and Giachetti, 1982), M_2 (M_{2a} , Mutschler et al., 1988; M_{2A} , Eglen and Whiting, 1986) and M_3 ($M_{2\beta}$, Mutschler et al., 1988; M_{2B} , Eglen and Whiting, 1982) according to Doods et al. (1987). Pirenzepine shows higher affinity for M_1 receptors than for the M_2 and M_3 types (Hammer and Giachetti, 1982), methoctramine displays high selectivity for the M_2 type (Melchiorre et al., 1987; Melchiorre, 1988; in press; Michel and Whiting, 1988), whereas hexahydro-sila-difenidol and 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP) show higher affinity for M_3 and M_1 types than for M_2 receptors (Eltze and Figala, 1988; Mutschler et al., 1988;

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Barlow et al., 1976). More recently, Lambrecht et al. (1988a, b, c) have developed the highly selective M_3 muscarinic antagonist, p-fluoro-hexahydro-sila-difenidol, as well as the very potent and selective M_1 muscarinic antagonists, o-methoxy-sila-hexocyclium and (R)-trihexyphenidyl.

The central administration of acetylcholine agonists not only has several effects on the regulation of body fluids and of blood pressure in the rat: stimulation of water intake, inhibition of salt appetite, release of vasopressin but in addition, produces a neurogenic hypertensive response (for review see Fitzsimons, 1979; Simpson, 1986). The understanding of the functional relationships between these effects can only be understood if the receptor subtype involved is known. Selective pharmacological manipulations of these functions also can only be carried out with knowledge of the subtype of receptor involved in each of the above mentioned effects. On the basis of the available evidence that drinking induced by acetylcholine agonists is mediated by muscarinic receptors (Fitzsimons, 1979), and taking advantage of the variety of selective muscarinic antagonists mentioned above, we designed the present study to investigate the subtype of muscarinic receptor involved in carbachol-induced drinking. An earlier investigation had provided indications that M_2 receptors may not be involved (Massi et al., 1989) in the response.

2. Materials and methods

2.1. Animals

Male albino Wistar rats (Charles River, Calco Como, Italy) averaging 250-275 g at the beginning of the experiments were housed individually in a temperature-controlled room on a 12:12 light-dark cycle. Food in pellets (MILL, Morini, Reggio Emilia, Italy) and tap water were available ad libitum.

2.2. Drugs

The following drugs were used: carbachol (carbamoylcholine chloride) (Merck), [Ile^5]angiotensin II (Peninsula Lab. Inc.); pirenzepine {5,11-

dihydro-11-[(4-methyl-1-piperazinyl)-acetyl]-6H-pyrido[2,3-b][1,4]benzodiazepin-6-one} dihydrochloride and (R)-trihexyphenidyl hydrochloride were kindly supplied by Dr. A. Giachetti and Dr. A.J. Aasen, respectively; methoctramine {N,N'-bis[6-[(2-methoxybenzyl)amino]hexyl]-1,8-octanediamine tetrahydrochloride, 4-DAMP (4-diphenyl-acetoxy-N-methylpiperidine methiodide), hexahydro-sila-difenidol [cyclohexylphenyl(3-piperidonopropyl) silanol hydrochloride], p-fluoro-hexahydro-sila-difenidol [cyclohexyl(4-fluorophenyl)(3-piperidinopropyl)silanol] and o-methoxy-sila-hexocyclium (4-{[cyclohexylhydroxy(2-methoxyphenyl)silyl]methyl}-1,1-dimethylpiperazinium methyl sulfate) were all synthesized in our laboratories.

2.3. Surgery and intracranial injections

All the animals were anesthetized (Equithesin, 3 ml/kg body weight i.p.) and fitted by stereotaxic surgery with a stainless-steel cannula (o.d. 600 μ m) aimed at 1 mm above the lateral ventricle. The guide cannula was attached to the skull by means of jewelry screws and dental acrylic cement. The rats were allowed one week to recover from surgery before testing began.

The drugs to be tested were dissolved in sterile isotonic saline, with the exception of hexahydro-sila-difenidol and p-fluoro-hexahydro-sila-difenidol that were dissolved in distilled water, being slightly soluble in isotonic saline. The drugs were given by intracerebroventricular (i.c.v.) injection through a stainless-steel injector (o.d. 300 μ m) temporarily inserted into the guide cannula and protruding 2 mm beyond the cannula tip. This technical expedient was adopted in order to reduce the possibility of back-flow of the solutions into the guide cannula. All the drugs were given in a constant volume of 1 μ l, except hexahydro-sila-difenidol and p-fluoro-hexahydro-sila-difenidol that were given in a 2- μ l volume.

2.4. Experimental procedure

2.4.1. Effect of muscarinic antagonists on carbachol-induced drinking

Carbachol (1 μ g/rat) was administered by i.c.v. injection to water-replete rats 1 min after the i.c.v.

administration of a muscarinic antagonist or of vehicle (controls). After carbachol injection, the animals were returned to their home cages where water and food were freely available. Water intake was recorded over a 2-h period of observation. For each antagonist, the experiment was carried out according to a 'within subject' design, in which each rat received all the doses of antagonists as well as the vehicle used for the antagonist itself. The rats were tested twice a week. Different groups of animals were used for the different antagonists.

2.4.2. Effect of muscarinic antagonists on angiotensin II-induced drinking

[Ile⁵]Angiotensin II was given by i.c.v. injection to water-replete rats at the dose of 100 ng/rat. Again, muscarinic antagonists or simple vehicle were administered i.c.v. 1 min before [Ile⁵]angiotensin II administration. Water intake was recorded for 60 min after the second i.c.v. injection. Only one dose of antagonist, the highest tested versus carbachol-induced drinking, was used. Each animal was also tested with the appropriate vehicle.

2.5. Statistical analysis

The data are reported as means \pm S.E.M. Statistical analysis of data for each compound tested was done by multifactorial analysis of variance (repeated measures) to check the overall significance of drug treatment. Planned pairwise comparisons were carried out by means of t-tests. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Effect of muscarinic antagonists on carbachol-induced drinking

Carbachol, given by pulse i.c.v. injection at a dose of 1 μ g/rat, induced copious drinking within 1 or 2 min following injection. Most of the dipsogenic response was expressed in the first 60-min period. However, additional drinking was also detected in several animals in the 2nd h of observation. The overall water intake in the 2-h period

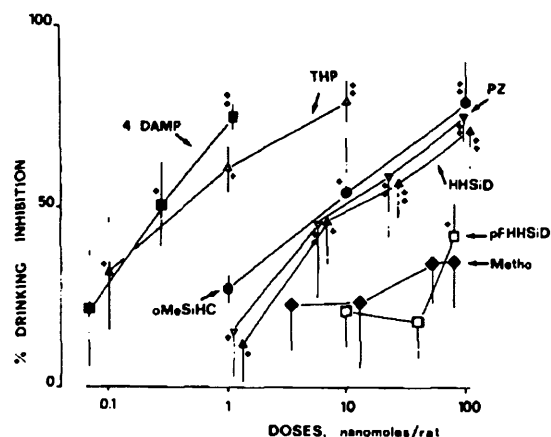


Fig. 1. Percent inhibition induced by different doses of 4-DAMP, (R)-trihexyphenidyl (THP), *o*-methoxy-sila-hexocyclium (*o*-MeSiHC), pirenzepine (PZ), hexahydro-sila-difenidol (HHSiD), *p*-fluoro-hexahydro-sila-difenidol (pFHHSiD) and methoctramine (Metho) of drinking induced by carbachol (1 μ g/rat). The values are means \pm S.E.M. for 6-10 subjects. The inhibitions reported were determined at 120 min after carbachol administration. Difference from controls (vehicle + carbachol): * $P < 0.05$; ** $P < 0.01$; where not indicated, the difference from controls was not statistically significant.

ranged between 8 and 12 ml of water/rat in the different experimental groups.

Figure 1 shows the dose-response relationships for the inhibitory effect of the muscarinic antagonists on carbachol-induced drinking. To compare the effects of these antagonists, water intake was expressed as percent of that of the controls and doses are reported in nmol/rat.

The potency (expressed as $-\log ID_{50}$) of the seven antagonists tested to inhibit carbachol-induced drinking is reported in table 1. In the range of doses tested, none of the muscarinic antagonists evoked evident behavioural alterations that might have prevented the animal from drinking.

As seen in previous experiments (Massi et al., 1989), the selective M₂ antagonist, methoctramine, produced a very slight inhibition of carbachol-induced drinking, in the range of doses from 3.3 to 80.3 nmol/rat (2.5-60 μ g/rat). The overall analysis of variance revealed the absence of a statistically significant drug effect, together with the absence of drug-time interaction. In the present study 60 μ g/rat was the highest dose of methoctramine tested, as we observed that administration of 100 μ g/rat plus carbachol 1 μ g pro-

TABLE 1

Comparison of $-\log ID_{50}$ values (with 95% confidence limits (c.l.)) of muscarinic antagonists tested on carbachol-induced drinking, with their pA_2 values at M_1 (rabbit vas deferens), M_2 (guinea-pig left atria) and M_3 (guinea-pig or rat ileum) receptors. Data taken from ^a Eltze and Figala (1988), ^b Melchiorre et al. (1987), ^c Lambrecht et al. (1988a), ^d Lambrecht et al. (1988c), ^e Lambrecht et al. (1988b), ^f Eltze (1988), and ^g Eglen and Whiting (1986). ^h Binding affinities (pK_i values) at $M_1/M_2/M_3$ receptors, (R)-trihexyphenidyl: 9.0/7.7/8.1; 4-DAMP: 9.1/8.1/8.9 (Waelbroeck et al., 1988; 1989)

Antagonist	$-\log ID_{50}$ (95% c.l.)	pA_2		
		M_1	M_2	M_3
Methoctramine	< 7.1	6.85 ^a	8.13 ^b	5.69 ^b
p-Fluoro-hexahydro-sila-difenidol	< 7.1	6.68 ^c	6.01 ^c	7.84 ^c
Pirenzepine	8.03 (7.63; 8.44)	8.24 ^c	6.82 ^c	6.88 ^c
Hexahydro-sila-difenidol	7.96 (7.41; 8.50)	7.92 ^c	6.53 ^c	7.96 ^c
o-Methoxy-sila-hexocyclium	8.13 (7.98; 8.28)	8.31 ^d	6.41 ^d	6.96 ^d
(R)-Trihexyphenidyl ^h	9.29 (7.90; 10.68)	10.11 ^e	8.15 ^e	8.68 ^e
4-DAMP ^h	9.55 (9.19; 9.90)	9.12 ^f	7.90 ^g	9.0 ^g

duced heavy prostration, followed by episodes of intense locomotor stimulation often ending in rotations along the rat's longitudinal axis. The M_3 -selective antagonist, p-fluoro-hexahydro-sila-difenidol, evoked a slight and statistically not significant inhibition of carbachol-induced drinking at doses up to 40 nmol/rat (15.4 μ g/rat). A significant drug effect was detected only in response to 80 nmol/rat ($F(1,7) = 14.27$; $P < 0.01$); pairwise comparisons showed that the inhibitory effect was significant throughout the 2-h period of observation. Even at this dose, however, the inhibition was less than 50%. Higher doses were not tested because of problems related to drug solubility. On the other hand, the five other muscarinic antagonists tested evoked a marked suppression of carbachol-induced drinking, the most potent being 4-DAMP and (R)-trihexyphenidyl. The overall analysis of variance revealed a powerful effect of 4-DAMP in the range of doses tested ($F(3,15) = 6.93$; $P < 0.005$). Following the lowest dose of 0.06 nmol/rat (31 ng/rat) the intake of treated rats was lower, but statistically indistinguishable from that of the controls. The inhibitory effect, on the other hand, was very pronounced and statistically significant at the dose of 0.276 and 1.10 nmol/rat. The time course of the drinking inhibition induced by 4-DAMP is shown in fig. 2. The ID_{50} of the drug on carbachol-induced drinking was 0.288 nmol (95% confidence limits (c.l.): 0.127-0.650). The selective M_1 antagonist, (R)-trihexyphenidyl,

was tested in the range of doses of 0.1, 1 and 10 nmol/rat (0.03-3.37 μ g/rat). The inhibitory effect at the lowest dose tested was significant up to 60 min following carbachol injection. The higher doses significantly inhibited drinking throughout the 2-h period of observation. The ID_{50} of the drug was 0.51 nmol (95% c.l.: 0.021-12.51). The M_1 -selective antagonist, o-methoxy-sila-hexocyclium, was about 10 times less potent than (R)-trihexyphenidyl. It did not evoke significant inhibition of drinking at the dose of 1 nmol/rat (0.47 μ g) but markedly suppressed drinking at 10 and

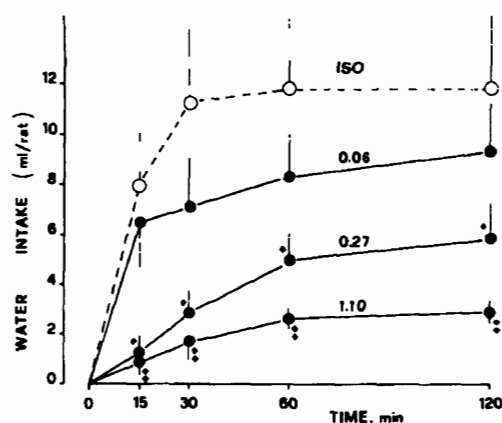


Fig. 2. Time course of drinking induced by carbachol (1 μ g/rat) following pretreatment with isotonic saline (ISO) or with different doses (nmol/rat) of 4-DAMP. Difference from controls: * $P < 0.05$; ** $P < 0.01$; where not indicated the difference from controls was not statistically significant.

100 nmol/rat. The ID_{50} of o-methoxy-sila-hexocyclium was 7.36 nmol (95% c.l.: 5.19-10.45). The M_1 -selective antagonist, pirenzepine, was slightly less potent than o-methoxy-sila-hexocyclium. In the range of doses tested, 1.17-94.2 nmol/rat (0.5-40 μ g/rat), it produced a highly significant effect ($F(4,20) = 36.64$; $P < 0.0001$). The effect was significant throughout the 2-h period of observation even at the dose of 1.17 nmol. The ID_{50} of pirenzepine was 9.31 nmol (95% c.l.: 3.66-23.67). Finally, hexahydro-sila-difenidol proved to be essentially as potent as pirenzepine, its ID_{50} being 11.09 nmol (95% c.l.: 3.15-39.03).

3.2. Effect of muscarinic antagonists on angiotensin II-induced drinking

To exclude the possibility that the muscarinic antagonists tested might have inhibited carbachol-induced drinking by producing a behavioural impairment, the same drugs were tested on angiotensin-induced drinking. For each drug we tested the highest dose used versus carbachol-induced drinking. Angiotensin II evoked a very prompt dipsogenic response, within 15-60 s. Most of the drinking occurred in the first 15 min after i.c.v. injection and was usually complete in the first 30 min in most of the rats treated. The water intake

after i.c.v. administration of each of the seven muscarinic antagonists tested was very similar and statistically indistinguishable from that of control rats receiving vehicle only (table 2).

4. Discussion

It is important to emphasize first that none of the muscarinic antagonists tested produced a significant inhibition of [Ile^5]angiotensin II-induced drinking, even at the highest doses employed, versus carbachol-induced drinking (table 2). This finding provides evidence that the inhibition of carbachol-induced drinking that we observed cannot be related to general behavioural impairment induced by the drugs, but is indeed the expression of a selective interaction with the cholinergic mechanism controlling drinking.

Concerning the characterization of the receptor subtype involved in carbachol-induced drinking, the present results suggest that M_1 receptors, rather than other subtypes of muscarinic receptors, apparently mediate the response. The possibility that M_2 receptor are involved is ruled out by the inactivity of the M_2 -selective muscarinic antagonist, methoctramine, in our test. This finding is in keeping with our previous results (Massi et al., 1989), as well as with the report of Schiavone et al. (1989) who used the M_2 antagonist, AF-DX 116. Moreover, the selective M_3 antagonist, p-fluoro-hexahydro-sila-difenidol, slightly inhibited drinking only at the very high dose of 80 nmol/rat, arguing against the involvement of an M_3 receptor. Finally, the observation that, as reported in table 1, the five antagonists that inhibited carbachol-induced drinking effectively showed a good correlation between inhibitory potency in our test and affinity for the M_1 receptor in rabbit vas deferens is direct evidence in favour of the mediation of the effect by an M_1 receptor. In fact: (a) pirenzepine, hexahydro-sila-difenidol and o-methoxy-sila-hexocyclium have pA_2 values for M_1 receptors clearly higher than those of methoctramine and of p-fluoro-hexahydro-sila-difenidol, which were clearly less potent to inhibit carbachol-induced drinking; (b) pirenzepine, hexahydro-sila-difenidol and o-methoxy-sila-

TABLE 2

Cumulative water intake in 60 min following i.c.v. injection of [Ile^5]angiotensin II (100 ng/rat) in rats pretreated with muscarinic antagonists (doses in nmol/rat) or with simple vehicle

Antagonist (dose)	Water intake (ml/rat) ^a	
	Vehicle	Antagonist
Methoctramine (80.3)	16.0 \pm 2.1	21.2 \pm 1.6
p-Fluoro-hexahydro-sila-difenidol (80)	15.5 \pm 1.2	17.2 \pm 2.1
Pirenzepine (94.2)	14.2 \pm 1.6	16.6 \pm 1.6
Hexahydro-sila-difenidol (108.6)	17.0 \pm 2.0	14.2 \pm 3.5
o-Methoxy-sila-hexocyclium (100)	16.1 \pm 2.8	15.1 \pm 1.6
(R)-Trihexyphenidyl (10)	14.1 \pm 3.2	17.2 \pm 1.0
4-DAMP (1.1)	17.1 \pm 2.5	15.2 \pm 2.0

^a The difference between vehicle- and antagonist-treated rats never reached statistical significance.

hexocyclium, which have very similar pA_2 values (from 7.92 to 8.31), proved to be essentially equipotent in our test; (c) 4-DAMP and (R)-trihexyphenidyl, which have pA_2 values for M_1 receptors higher than those of the previously mentioned antagonists, were also more potent in our test. Indeed, 4-DAMP proved to be slightly more potent than (R)-trihexyphenidyl although its affinity for M_1 receptors is about one order of magnitude lower in functional studies (table 1). However, in binding experiments using NB-OK 1 cells, 4-DAMP and (R)-trihexyphenidyl competed equipotently with N-methylscopolamine at M_1 receptors ($pK_i = 9.1$ and 9.0 , respectively) (Waelbroeck et al., 1988; 1989). The pA_2 values for M_2 and M_3 receptors of the seven antagonists tested are not related to their potency for inhibition of carbachol-induced drinking (table 1).

The conclusion from the present study is not consistent with that drawn from a previous one (Hagan et al., 1987) which suggested that M_2 and/or M_3 receptors would mediate cholinergic drinking in the rat. Indeed, the results in this report are not strictly comparable to ours, since these authors injected their substances directly into the lateral hypothalamus adjacent to the perifornical region, while our drugs were injected into the ventricular space. Probably, following i.c.v. injection, drugs reach only to a slight extent the lateral hypothalamus while they can act at other sites such as the subfornical organ, which is very sensitive to the dipsogenic action of carbachol and, also is easily accessible from the ventricular space. However, the different conclusion reached might have been a consequence of differences in pharmacological tools employed. In fact, Hagan et al. (1987) based their conclusions largely on the findings obtained with muscarinic agonists, while we used only antagonists. Our choice was related to the fact that putative M_1 agonists exhibit low efficacy and behave as partial agonists in a range of pharmacological and biochemical tests. Differences in efficacy due to variations in intrinsic activity or efficiency of receptor coupling, may give a false impression of receptor specificity. Moreover, our study involved a greater variety of antagonists, including the most recent and selective M_2 and M_3 antagonists, methoctramine and

p-fluoro-hexahydro-sila-difenidol, which gave clear-cut support to our hypothesis of the involvement of M_1 receptors in cholinergic-induced drinking.

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