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Inhibition of cortical acetylcholine release and cognitive performance by histamine H_3 receptor activation in rats

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1 The effects of histamine and agents acting at histamine receptors on spontaneous and 100 mM K^+-evoked release of acetylcholine, measured by microdialysis from the cortex of freely moving rats, and on cognitive tests are described.

2 Local administration of histamine (0.1 – 100 μM) failed to affect spontaneous but inhibited 100 mM K^+-stimulated release of acetylcholine up to about 50%. The H_3 receptor agonists (**R**)-α-methylhistamine (RAMH) (0.1 – 10 μM), imetit (0.01 – 10 μM) and immepip (0.01 – 10 μM) mimicked the effect of histamine.

3 Neither 2-thiazolylethylamine (TEA), an agonist showing some selectivity for H_1 receptors, nor the H_2 receptor agonist, dimaprit, modified 100 mM K^+-evoked release of acetylcholine.

4 The inhibitory effect of 100 μM histamine was completely prevented by the highly selective histamine H_3 receptor antagonist, clobenpropit but was resistant to antagonism by triprolidine and cimetidine, antagonists at histamine H_1 and H_2 but not H_3 receptors.

5 The H_3 receptor-induced inhibition of K^+-evoked release of acetylcholine was fully sensitive to tetrodotoxin (TTX).

6 The effects of intraperitoneal (i.p.) injection of imetit (5 mg kg^{-1}) and RAMH (5 mg kg^{-1}) were tested on acetylcholine release and short term memory paradigms. Both drugs reduced 100 mM K^+-evoked release of cortical acetylcholine, and impaired object recognition and a passive avoidance response.

7 These observations provide the first evidence of a regulatory role of histamine H_3 receptors on cortical acetylcholine release *in vivo*. Moreover, they suggest a role for histamine in learning and memory and may have implications for the treatment of degenerative disorders associated with impaired cholinergic function.

Keywords: Acetylcholine; histamine; histamine H_3 receptor; frontoparietal cortex; microdialysis; object recognition; passive avoidance response

Introduction

There is increasing support for Green's (1964) early suggestion that histamine functions in brain and it is now unequivocally established that histamine acts as a neurotransmitter in the brain (Schwartz *et al.*, 1991; Wada *et al.*, 1991). Histamine H_3 receptors, initially detected as autoreceptors inhibiting histamine release from (Arrang *et al.*, 1983) and synthesis in (Arrang *et al.*, 1987b) rat cortical slices, also modulate the *in vitro* release of [^{3}H]-5-hydroxytryptamine (Schlicker *et al.*, 1988), [^{3}H]-noradrenaline (Schlicker *et al.*, 1989), [^{3}H]-dopamine (Schlicker *et al.*, 1993) and [^{3}H]-acetylcholine ([^{3}H]-ACh) (Clapham & Kilpatrick, 1992) from the rodent central nervous system (CNS). Although autoradiographic studies have shown that the presence of H_3 receptors is not restricted to histaminergic neurones (Martinez-Mir *et al.*, 1990; Cumming *et al.*, 1991; Pollard *et al.*, 1993), the modulation of 5-hydroxytryptaminergic and catecholaminergic tone was not confirmed by *in vivo* studies (Oishi *et al.*, 1990), and H_3 receptor activation did not inhibit [^{3}H]-ACh release from rat entorhinal cortex synaptosomes (Arrang *et al.*, 1995).

There is much evidence of central cholinergic system involvement in the modulation of learning and memory (Haroutunian *et al.*, 1985). Moreover, animal performance in cognitive tests can be altered by a variety of neurotransmitters which possibly influence cholinergic functions (Decker *et al.*, 1991). A histamine-ACh interaction in the brain is suggested by the observation that (**R**)-α-methylhistamine (RAMH), an H_3 receptor agonist (Arrang *et al.*, 1987a), antagonized the scopolamine-induced memory deficit in spatial learning (Smith *et al.*, 1994), which requires an intact hippocampus. Indeed, this finding is surprising, since RAMH decreased, and thioperamide, an H_3 receptor antagonist (Arrang *et al.*, 1987a), increased ACh release from the hippocampus of anaesthetized rats (Mochizuki *et al.*, 1994). Although these effects were weak and short-lasting (Mochizuki *et al.*, 1994), one might have envisaged that reduced availability of ACh in the synaptic cleft would impair cognitive performances, as recently suggested (Quirion *et al.*, 1995).

The present study was designed to evaluate the capacity of histamine to modulate ACh release from the frontoparietal cortex of freely-moving rats and to characterize the underlying mechanisms. Also, the possibility of an H_3 receptor-mediated mechanism in cognitive functions was investigated by assessing the effects of H_3 receptor agonists on object recognition and a passive avoidance response. These tasks are impaired by cholinergic blockade (Spignoli & Pepeu, 1987; Ennaceur & Meliani, 1992), and serve to measure a form of episodic memory, possibly localized in the frontal cortex (Goldman-Rakic, 1987). Some of these results were reported in abstract form (Blandina *et al.*, 1996).

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Methods

Animal housing and surgery

Male Wistar rats (225–275 g b.wt.) were housed in groups of three in a temperature-controlled room (20–24°C), allowed free access to food and water, and kept on a 12 h light/dark cycle. The rats were anaesthetized with chloral hydrate (400 mg kg^{-1}, i.p.) and placed in a stereotaxic frame (Stoelting, Stellar). Microdialysis tubes were inserted transversally in the frontoparietal cortex. The transversal microdialysis tubes were made of AN 69 membrane (Dasco, Italy), 220 μm internal diameter and 310 μm external diameter, molecular weight cut-off ⩾15,000 Da. The membrane, mounted on a guide wire of tungsten-stainless steel, was covered with Super-Epoxy glue except for an 8 mm section in the frontoparietal cortex. A thin stainless steel cannula (22 gauge, 1 cm long) was glued to the distal end of the tubing. After a sagittal incision, the overlying skin and the temporal muscles were retracted, and holes for placement of the dialysis tube were drilled bilaterally at coordinates: AP −0.5 mm and H 2.0 mm from bregma. All coordinates (Paxinos & Watson, 1982) were measured on the bone surface and referred to bregma, with bregma and lambda on a horizontal plane. One end of the guide wire of the microdialysis tubing was fixed to the micromanipulator of the stereotaxic instrument, and gently pushed through the brain. The exposed portion of membrane was positioned precisely by means of two reference marks corresponding to the outer surface of the temporal bones. Another steel cannula (22 gauge, 1 cm) was secured to the end of the dialysis tubing, the inner guide wire was removed. The cannulae, bent upward, were secured to the parietal bone with acrylic dental cement and the skin sutured. All surgical manipulations were performed under deep anaesthesia. Rats were then replaced in their home cages (1 rat per cage) to recover from surgery.

Microdialysis experiments

Twenty-four hours after surgery, each rat was placed in a Plexiglas cage. The inlet of the microdialysis probe was connected to a microperfusion pump (Carnegie Medicine, mod. CMA/100, Sweden) and the outlet was inserted into a 200 μl test tube containing 5 μl of 0.5 mM HCl to prevent hydrolysis of acetylcholine. The microdialysis tubing was perfused at the rate of 3 μl min^{-1} with Ringer solution (composition, mM: NaCl 147, $CaCl_2$ 1.2, KCl 4.0, pH 7.0). To recover detectable dialysate concentrations of acetylcholine, a cholinesterase inhibitor (physostigmine sulphate, 7 μM) was included in the perfusion solution. The molecular weight cut-off of the membrane allowed low molecular weight solutes to cross the dialysis membrane according to their concentration gradients. Hence, both the collection of endogenous molecules, and the administration of exogenous compounds were feasible.

After an equilibration period of 60 min, 30 μl fractions (resulting from 10-min intervals of perfusion) were collected. Rats were stimulated twice by a 10-min exposure to a 100 mM K^+-containing medium, given through the dialysis fibre, at 40 (S_1) and 140 min (S_2) after the equilibration period. Tonicity was maintained by reduction of the Na^+ concentration. The first stimulation period (S_1) was used as control; drugs, alone or in combination, were added to the perfusing medium 10 min before S_2 and maintained during the S_2 stimulation. Although the exact amount of drug infused into the tissue is unknown, it can be estimated as approximately 10–15%.

Accurate placement of microdialysis membrane was verified *post mortem* by gross visualization of coronal sections cut through and on either side of the probe path. Data from rats in which the membrane was not correctly positioned (fewer than 10% of the animals) were discarded.

All experiments were done in strict compliance with the recommendations of the EEC (86/609/CEE) for the care and use of laboratory animals and were approved by the Animal Care Committee of the Department of Pharmacology of the Universitá di Firenze.

h.p.l.c. method

Acetylcholine was determined by h.p.l.c.-electrochemical detection (Giovannini *et al.*, 1994). The h.p.l.c. apparatus consisted of a pump (Mod. 1350, BioRad, Richmond, CA, U.S.A.), a presaturation column (Chromspher 5 C18, 100 × 3 mm, Chrompack, Middleburg, NL), an injector (Mod. 7125, Rhehodyne, Cotati, U.S.A.), a guard column (reverse phase), an analytical column (Chromspher 5 C18, 100 × 3 mm, Chrompack), an enzyme reactor (10 × 2.1 mm, Chrompack), an electrochemical detector (Mod. LC4C, BioAnalytical System, West Lafayette, IN, U.S.A.) and a Perkin Elmer chart recorder. The analytical column was transformed into a cation exchange column by loading it with sodium lauryl sulphate (0.5 mg ml^{-1}). The enzyme reactor consisted of a 1-cm long column containing Lichrosorb-NH_2 activated with glutaraldehyde, and to which acetylcholinesterase (E.C. 3.1.1.7) and choline oxidase (E.C. 1.1.3.17) were covalently bound. The mobile phase had the following composition (mM): K_2HPO_4–KH_2PO_4 100, KCl 5, tetramethylammonium 1 and EDTA 0.3 (pH 8.0). Acetylcholine was separated on the cation exchange column. ACh was hydrolyzed by acetylcholinesterase to form acetate and choline in the postcolumn enzyme reactor, then choline was oxidized by choline oxidase to produce betaine and hydrogen peroxide. Hydrogen peroxide was detected by a platinum electrode with the potential set at 0.5 V. The flow rate was 0.75 ml min^{-1}. Peaks were identified by comparison of their retention times with those of the standards.

Quantification of acetylcholine

The levels of ACh in the perfusates were calculated by comparison of sample peak heights with external standard peak height and expressed as pmol 10 min^{-1}. Calibration curves for ACh were constructed by plotting the heights of peaks against the concentrations. Regression lines were then calculated and determination of unknown samples was carried out by the method of inverse prediction. The sensitivity limit was 500 fmol. Evoked release at the S_1 and S_2 periods of stimulation was calculated as the total minus the spontaneous release. Spontaneous release was obtained by averaging ACh content in the four samples immediately before the first K^+ stimulation (S_1). Drug effects were evaluated by calculating the ratio of the evoked release (S_2/S_1) for the two stimulation periods.

Behaviour

Object recognition (Ennaceur & Delacour, 1988) measures a form of short-term memory based on short and unrepeated experiments without any reinforcement, such as food or electric shocks. Object recognition is a one-trial task, and does not involve learning of any type, being based entirely on the spontaneous exploratory behaviour of rats towards objects. The apparatus is a white coloured polyvinyl chloride box (70 × 60 × 30 cm) with a grid floor which is easily cleaned, lighted by a 75 W lamp suspended 50 cm above the box. The objects to be discriminated were grey polyvinyl chloride shapes: cubes of 8 cm side, pyramids and cylinders of 8 cm height. The day before testing the rats were allowed to explore the box for 2 min. On the day of the test, two trials were given. The inter-trial interval was 60 min. In the first trial, two identical objects were put in two opposite corners of the box, and the time (t1) required by each rat to complete a cycle of 20 s of object exploration was recorded. Exploration was considered to be directing the nose at a distance less than 2 cm from the object and/or touching it with the nose. During the second trial (T2) one of the objects presented in the first trial was replaced by a new object of different shape, and rats were

left in the box for 5 min. The exploration periods of the familiar (tF) and the new object (tN) were recorded separately, and a discrimination index (D) was calculated according to the formula tN−tF/tN+tF. Care was taken to avoid place preference and olfactory stimuli by randomly changing the role (familiar or new object) and the position of the two objects during T2, and cleaning them carefully. Object recognition was carried out in an insulated room to avoid any noise which could impair the performance of the rats.

Passive avoidance response is a paradigm to evaluate short-term memory in experimental animals. The apparatus consists of two compartments (24 × 21 × 21 cm each) with grid floors which can be elecrified separately. The first compartment is a transparent Plexiglas box illuminated by a 100-W lamp, the second is a dark compartment with walls painted black. A guillotine door connects the two compartments. In the training trial, each rat was placed in the illuminated compartment, facing the guillotine door and, after a 30-s orientation period, the door was raised manually. As soon as the animal placed all four paws in the dark compartment, the guillotine door was lowered. The time elapsed before entering this chamber was recorded, and 0.6–0.8 mA shock (5 Hz, 20 ms) was delivered to the grid floor for 5 s. The intensity of the shock was selected after establishing the sensitivity threshold that produces the minimum vocalization and jumping responses. Immediately after receiving the shock, the rat was removed from the dark chamber, returned to its home cage, and the chambers were cleaned from faeces and urine. Retesting was performed 24 h after training. The rat was placed again in the illuminated compartment and the latency between door opeining and entry into the dark compartment was measured. Better performance is indicated by longer latencies (up to a maximum time of 120 s).

Analysis of data

The IC_{50} values of histamine and H_3 receptor agonists were calculated. A four parameter logistic equation was fitted ($R=0.99$) to the mean values of S_2/S_1. The equation used was:

$$R = R_i - (R_i - R_0)/[([A]/IC_{50})n_H + 1]$$

where R represents S_2/S_1 value in the presence of a specified concentration of agonist, [A]; R_0 represents S_2/S_1 value in the absence of agonist; R_i represents S_2/S_1 value at supramaximal concentrations of agonist; IC_{50} represents the concentration of agonist that produced a half-maximal inhibition, $(R_0+R_i)/2$, and n_H was the slope factor.

All values are expressed as means ± s.e.mean, and the number of experiments (n) is also indicated. Comparisons between two means were carried out by Student's t test, paired or unpaired as appropriate. When experiments involved more than two treatment groups the presence of significant treatment effects was first determined by a one-way analysis of variance (ANOVA) followed by Scheffe's test. For all statistical tests, $P<0.05$ was considered significant.

Drugs

The substances used in this study included histamine hydrochloride, tetrodotoxin, tetramethylammonium (Sigma Chemical Company Ltd., U.K.); dimaprit dihydrochloride, 2-thiazolylethylamine dihydrochloride (SmithKline Beecham, Surrey, U.K.); (**R**)-α-methylhistamine dihydrochloride, physostigmine sulfate (R.B.I., Natick, MA, U.S.A.); imetit dihydrobromide, immepip dihydrobromide, clobenpropit dihydrobromide were provided by R. Leurs and H. Timmerman. All other reagents and solvents were of h.p.l.c. grade or the highest grade available (Sigma Chemical Company Ltd., U.K.).

Results

Spontaneous and 100 mM K^+-evoked release of ACh from cortex of freely moving rats

Twenty-four hours after implantation with dialysis fibre, and after 60 min of perfusion the rat cortex spontaneously released ACh at stable rates, 3.51 ± 0.16 pmol 10 min^{-1} ($n=123$). Spontaneous relese of ACh was calculated for each experiment by averaging the mean of the four initially collected 10-min samples of perfusate, and did not decrease significantly with time. A first depolarization, induced by perfusing the cortices for 10 min with a medium containing 100 mM K^+, strongly stimulated the release of ACh (8.12 ± 0.37 pmol 10 min^{-1}, $n=123$). The maximal effect was achieved in the 10-min fraction collected during the perfusion with K^+-medium. The level of spontaneous ACh release was restored after K^+-medium was replaced with Ringer solution. During the K^+ pulse the rats appeared to be more active but did not show any abnormal behaviour.

In a subset of 7 control experiments a second identical 100 mM K^+ perfusion (S_2) was conducted 90 min after the first, when ACh release returned to basal values (Figure 1). The two consecutive exposures to 100 mM K^+ for 10 min released similar amounts of ACh. The mean S_2/S_1 ratio was 1.38 ± 0.10 (Figure 1).

H_3 but not H_1 or H_2 receptor agonists inhibited 100 mM K^+-evoked release of ACh

Inclusion of histamine (100 μM) in the perfusing Ringer solution for 30 min produced no significant modification of the amounts of spontaneously released ACh. The spontaneous

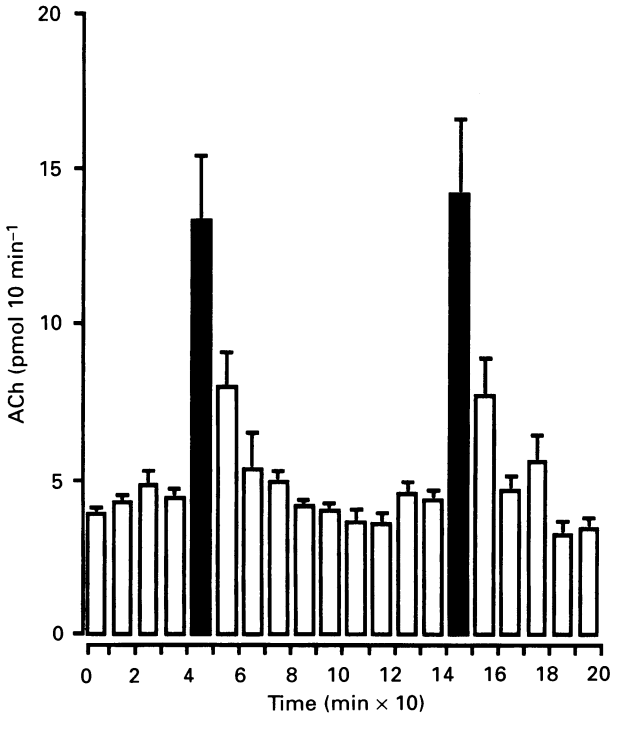

Figure 1 Spontaneous (open columns) and K^+-evoked (solid columns) release of acetylcholine (ACh) from the cortex of freely moving rats. ACh was measured in fractions collected every 10 min beginning 60 min after the onset of the perfusion. At 40 (S_1) and 140 (S_2) min the perfusion medium was changed from 4 to 100 mM KCl for 10 min. Isotonicity was maintained by reducing NaCl concentration. Spontaneous release of ACh averaged 4.3 ± 0.6 pmol 10 min^{-1}. Spontaneous release of ACh was calculated for each experiment by averaging the mean of the four initially collected 10-min samples of perfusate. Shown are means ± s.e.mean of 7 experiments.

release of ACh averaged 4.4±0.1 pmol 10 min^{-1} in the absence, and 4.4±0.8 pmol 10 min^{-1} in the presence of the amine ($n=3$). However, addition of histamine to the perfusing medium 10 min before S_2 and during S_2 stimulation, resulted in a significant inhibition of the ACh release evoked by 100 mM K^+ (Figure 2). The inhibition was concentration-dependent (0.1–100 μM), with a maximal decrease of about 50% (Figure 2). The H_3 receptor agonists RAMH, 0.1–10 μM; imetit, 0.01–10 μM; (Garbarg *et al.*, 1992; Howson *et al.*, 1992; Van der Goot *et al.*, 1992) and immepip, 0.01–10 μM; (Vollinga *et al.*, 1994) mimicked the inhibitory effect of histamine (Figure 2). Since each rat was exposed to only a single concentration of agonist, the IC_{50} values of histamine, RAMH, imetit and immepip were calculated by averaging S_2/S_1 values in three to six experiments at each concentration. Histamine (110 nM) and RAMH (80 nM) showed slightly lower potency than imetit (25 nM) and immepip (34 nM).

A significant inhibition of 100 mM K^+-evoked release of ACh was also observed following intraperitoneal (i.p.) injection of 5 mg kg^{-1} of RAMH and of imetit (Figure 3). Because of the *in vivo* time course of the effects of i.p. RAMH (Arrang *et al.*, 1987a) and imetit (Garbarg *et al.*, 1992) on cerebral histamine turnover, RAMH was injected 30 min, and imetit 120 min before S_2. Saline (250 μl) was injected i.p. to control animals 30 min before S_2. Each drug was freshly prepared and diluted in saline to permit the injection of a constant volume of 1 ml kg^{-1} to each rat.

In contrast to the response to histamine, H_3 receptor agonists, 100 μM 2-thiazolylethylamine (TEA), a compound showing some selectivity for H_1 receptors (Ganellin, 1982), and 10 μM dimaprit, a selective H_2 receptor agonist (Parson *et al.*, 1977), added to the perfusing medium 10 min before S_2 and maintained during the S_2 stimulation, had no significant effect on 100 mM K^+-evoked ACh release. The mean S_2/S_1 ratio was 1.17±0.13, in the presence of TEA ($n=6$), 1.32±0.07 in that of dimaprit ($n=3$). These values were not significantly different from the S_2/S_1 ratio observed in the absence of drugs.

H_3 but not H_1 or H_2 receptor antagonists blocked the inhibition of 100 mM K^+-evoked release of ACh produced by 100 μM histamine

The histamine receptor antagonists, alone and together with 100 μM histamine, were added to the perfusing medium 10 min before S_2 and maintained during the S_2 stimulation. The H_3 receptor antagonist, clobenpropit (Van der Goot *et al.*, 1992) alone was without effect on 100 mM K^+-evoked ACh release (Figure 4), but, at increasing concentrations in the nanomolar range, progressively antagonized the inhibition of 100 mM K^+-evoked ACh release produced by 100 μM histamine (Figure 4).

Conversely, neither 100 nM triprolidine, antagonist of the H_1 receptor (Ison *et al.*, 1971), nor 100 μM cimetidine, an H_2 receptor antagonist (Durant *et al.*, 1977), prevented 100 μM histamine-elicited inhibition of 100 mM K^+-evoked ACh release (Figure 4). In the absence of histamine both failed to alter 100 mM K^+-evoked ACh release. The mean S_2/S_1 ratio was 1.31±0.11 in the presence of 100 nM triprolidine ($n=3$), and 1.27±0.12 in the presence of 100 μM cimetidine ($n=3$).

Effects of combined H_1 and H_2 receptor blockade on the inhibition of 100 mM K^+ evoked release of ACh produced by histamine

The effect of the combination of 100 nM triprolidine and 100 μM cimetidine on the inhibition of 100 mM K^+-evoked ACh release produced by two concentrations of histamine, 1 and 0.1 μM, was examined. The differences between the means of the groups reached statistical significance ($F=5.01$; $P=0.11$;

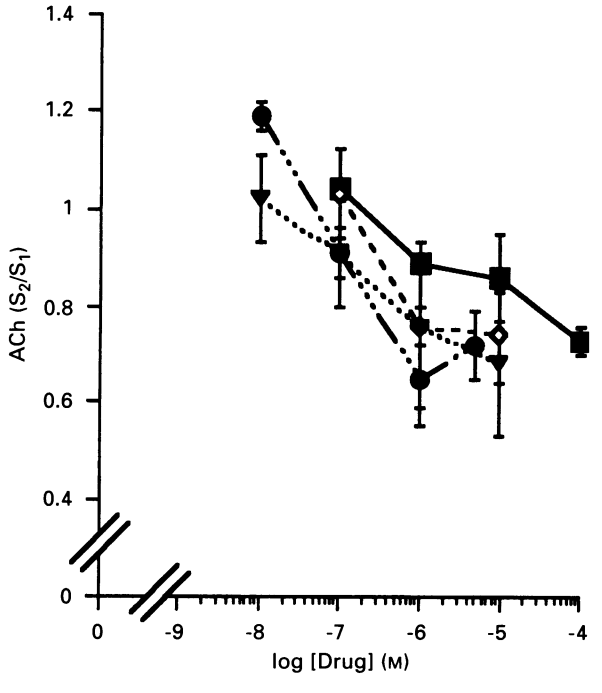

Figure 2 Influence of local administration of histamine and histamine H_3 receptor agonists on 100 mM K^+-evoked release of acetylcholine (ACh) from the cortex of freely moving rats. The perfusion medium was changed from 4 to 100 mM KCl for 10 min at 40 (S_1) and 140 (S_2) min after equilibration. Histamine or histamine H_3 receptor agonists were added 10 min before S_2 stimulation to the perfusion medium and remained throughout the S_2 stimulation. Each rat was exposed to only one concentration of agonist. Histamine: (■); RAMH (◇); imetit (▼) and immepip (●). Each point represents the mean value±s.e.mean of 3 to 6 experiments. Histamine 1 μM: $P=0.014$; 10 μM: $P=0.009$; 100 μM: $P<0.001$; RAMH 1 μM: $P=0.019$; 10 μM: $P<0.001$; imetit 1 μM: $P=0.003$; 10 μM: $P<0.001$; immepip 1 μM: $P=0.003$; 5 μM: $P=0.007$; as compared with 100 mM K^+ in the absence of histamine and H_3 receptor agonists (analysis of variance and Scheffe's test).

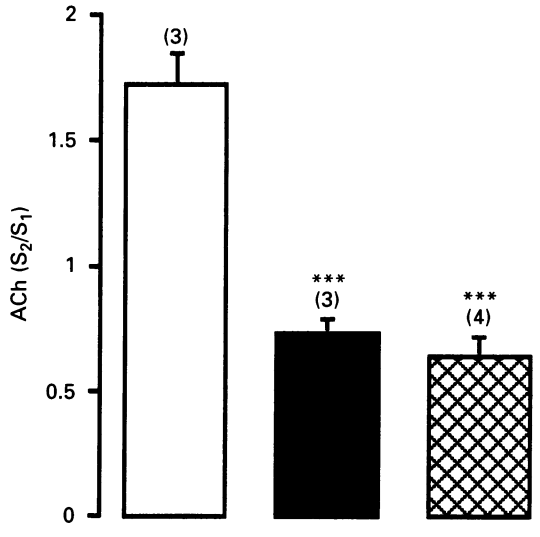

Figure 3 Influence of systemic administration of the H_3 receptor agonists RAMH and imetit on 100 mM K^+-evoked release of acetylcholine (ACh) from the cortex of freely moving rats. Rats were injected i.p. with RAMH (5 mg kg^{-1}) 30 min, and imetit (5 mg kg^{-1}) 120 min prior to S_2. Each drug was freshly prepared and diluted in sterile saline to permit the injection of a constant volume of 1 ml kg^{-1} to each rat. Controls were injected i.p. with saline (250 μl) 30 min before S_2. Controls: open column; RAMH: solid column; imetit: cross-hatched column. Shown are means± s.e.mean with number of experiments in parentheses. ***$P<0.001$ vs. control (analysis of variance and Scheffe's test).

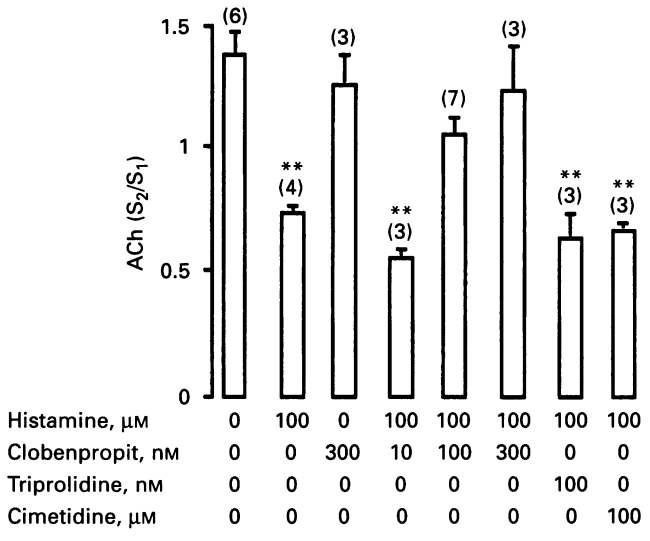

Figure 4 Influence of histamine H_1, H_2 and H_3 receptor antagonists on 100 μM histamine-induced inhibition of 100 mM K^+-evoked acetylcholine (ACh) release from the cortex of freely moving rats. The perfusion medium was changed from 4 to 100 mM KCl for 10 min at 40 (S_1) and 140 (S_2) min after equilibration. Histamine H_1, H_2 and H_3 receptor antagonists, alone or in combination with 100 μM histamine, were added 10 min before S_2 stimulation to the perfusion medium and maintained throughout the S_2 stimulation. Shown are means ± s.e.mean with number of experiments in parentheses. $^{**}P<0.01$ vs. control (analysis of variance and Scheffe's test).

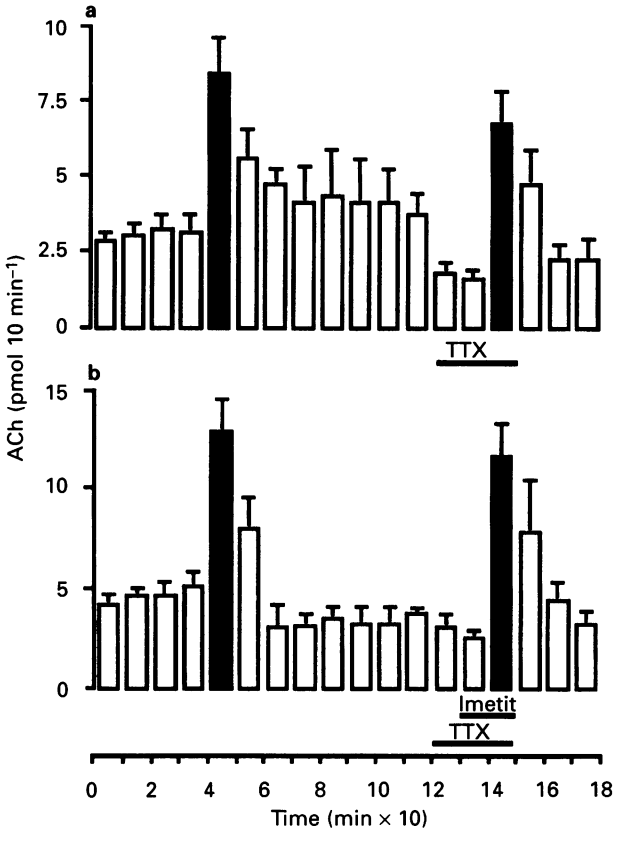

Figure 5 Spontaneous (open columns) and K^+-evoked (solid columns) release of acetylcholine (ACh) from the cortex of freely moving rats in the presence of 0.5 μM TTX alone (a) and in combination with 10 μM imetit (b). At 40 (S_1) and 140 (S_2) min the perfusion medium was changed from 4 to 100 mM KCl for 10 min. Isotonicity was maintained by reducing NaCl concentration. Horizontal bars show the duration of perfusion with 0.5 μM TTX (a and b) and 10 μM imetit (b). Shown are means ± s.e.mean of 6 (a) and 4 (b) experiments.

ANOVA). Scheffe's *post-hoc* analysis showed no significant change in the inhibitory response to 1 μM histamine, the mean S_2/S_1 ratio being 0.89 ± 0.07 ($n=5$) in the absence, and 0.74 ± 0.09 ($n=7$) in the presence of triprolidine and cimetidine. However, when both antagonists were present, a significant greater inhibitory effect of 0.1 μM histamine was observed, the mean S_2/S_1 ratio being 1.01 ± 0.06 ($n=7$) in the absence, and 0.58 ± 0.12 ($n=4$) in the presence of the antagonists ($P=0.02$).

H_3 receptor-elicited inhibition of 100 mM K^+ evoked release of ACh was abolished in the presence of tetrodotoxin

Tetrodotoxin (TTX) a voltage-dependent Na^+-channel blocker, 0.5 μM, was infused directly into the cortex through the dialysis fibre 20 min before S_2 and maintained during S_2 stimulation alone (Figure 5a), or in the presence of 10 μM imetit (Figure 5b). By pooling all ten experiments with TTX, ACh spontaneous release averaged 3.67 ± 0.4 pmol 10 min^{-1}, and ACh spontaneous release in the presence of TTX, calculated for each experiment by averaging the mean of the two 10-min samples of perfusate containing TTX and collected immediately before S_2, was 2.45 ± 0.36 pmol 10 min^{-1} (Figure 5a and b). Because TTX significantly decreased spontaneous ACh release by more than 40% ($P=0.009$, Student's paired t test; $n=10$), 100 mM K^+-evoked release of ACh for S_2 was calculated as the difference between ACh release measured during S_2 and the immediately preceding spontaneous ACh release in the presence of TTX. The exposure to 100 mM KCl released similar amounts of ACh in the absence and in the presence of 0.5 μM TTX (S_2/S_1 : 1.1 ± 0.1; $n=6$) (Figure 5a). However, in the presence of TTX, 10 μM imetit, a concentration that can markedly reduce K^+-evoked release of ACh (Figure 2), failed to inhibit the release of ACh (S_2/S_1 : 1.1 ± 0.12; $n=4$) (Figure 5b). Thus, perfusion of the cortices for 20 min with 0.5 μM TTX failed to modify 100 mM K^+-evoked release of ACh as compared to controls, but abolished completely the inhibition induced by 10 μM imetit.

Object recognition was impaired by H_3 receptor agonists

Dosing schedules for H_3 receptor agonists in behavioural experiments paralleled those for effects of systemic agonists on K^+-evoked cortical ACh release. RAMH (5 mg kg^{-1}) was injected i.p. 30 min, and imetit (1 and 5 mg kg^{-1}) 120 min prior to the training trial. Each drug was freshly prepared and diluted in saline to permit the injection of a constant volume of 1 ml kg^{-1} to each rat. Saline was injected i.p. to control animals 30 min prior to the training trial. Analysis of the exploration time (t1) during the training trial failed to reveal any significant group effect (Table 1). In T2 control rats spent significantly more time exploring the new object than the familiar one (Table 1). Animals treated with 1 mg kg^{-1} imetit were comparable in their performance with control rats. Those treated with 5 mg kg^{-1} imetit, however, showed no significant difference in the exploration time of the new object compared to that of the familiar one with a concomitant significant reduction in the discrimination index (D), decreased significantly from 0.40 ± 0.04 (control) to 0.03 ± 0.03 (5 mg kg^{-1} imetit) (Table 1). Rats treated with RAMH similarly did not show any significant difference in the exploration time of the new object as compared to that of the familiar one, and the discrimination index (D) was 0.11 ± 0.07 (Table 1).

Passive avoidance response was impaired by H_3 receptor agonists

Figure 6 displays the effects of i.p. administration of imetit and RAMH on passive avoidance test performance. Each drug was

Table 1 Effects of imetit and RAMH on object recognition test

Drugs	n	T2 t1 (s)	T2 tF (s)	T2 tN (s)	D
Saline 250 μl	28	214±12	6.2±0.8	13.4±1.4[a]	0.40±0.04
Imetit 1 mg kg^{-1}	13	175±12	5.9±0.7	13.3±1.6[b]	0.37±0.05
Imetit 5 mg kg^{-1}	15	182±15	9.2±1.1	9.9±1.3	0.03±0.03[c]
RAMH 5 mg kg^{-1}	14	255±14	3.6±0.6	4.4±0.8	0.11±0.07[c]

In the first trial, the time (t1) required by each rat to complete a cycle of 20 s of object exploration was recorded. During the second trial (T2), given 60 min after the first trial, one of the objects presented in the first trial was replaced by a new object of different shape, and rats were left in the box for 5 min. The exploration periods of the familiar (tF) and the new object (tN) were recorded separately, and a discrimination index (D) was calculated according to the formula tN−tF/tN+tF.
Values represent the mean±s.e.mean of (*n*) experiments. [a]Different from correspondent tF ($P<0.0001$, Student's paired *t* test); [b]Different from correspondent tF ($P<0.0001$, Student's paired *t* test); [c]Different from Saline D ($P<0.0001$, Scheffe's test).

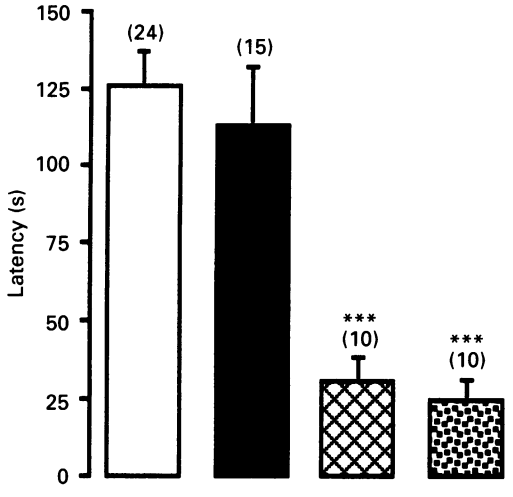

Figure 6 Effects of imetit and RAMH on the 24 h retention of a passive avoidance conditioned response. Rats were injected i.p. with RAMH (5 mg kg^{-1}) 30 min, and imetit (1 and 5 mg kg^{-1}) 120 min before the training trial. Each drug was freshly prepared and diluted in sterile saline to permit the injection of a constant volume of 1 ml kg^{-1} to each rat. Controls were injected i.p. with saline (250 μl) 30 min before the training trial. Crossthrough latencies of the training trial of the four groups were indistinguishable from each other. Retesting was performed 24 h after training. Controls: open column; imetit, 1 mg kg^{-1}: solid column; imetit, 5 mg kg^{-1}: cross-hatched column; RAMH, 5 mg kg^{-1}: stippled column. Shown are means±s.e.mean with number of experiments in parentheses. ***$P<0.001$ vs. saline (analysis of variance and Scheffe's test).

diluted in saline to permit the i.p. injection of a constant volume of 1 mg kg^{-1} to each rat. Saline and RAMH were administered i.p. 30 min, and imetit 120 min prior to the training trial. Analysis of variance on the crossthrough latencies revealed a significant treatment effect ($F=14.1$, $P<0.0001$). Scheffe's *post-hoc* analysis showed that latencies of rats receiving RAMH and the 5 mg kg^{-1} dose of imetit were significantly different from those of rats receiving saline ($P=0.0002$, and $P=0.0006$, respectively), and of rats receiving the 1 mg kg^{-1} dose of imetit ($P=0.009$, and $P=0.004$, respectively). No other comparison was significant. Thus RAMH and imetit, at doses of 5 mg kg^{-1}, impaired passive avoidance behaviour, while 1 mg kg^{-1} of imetit was ineffective.

Discussion

Histamine interacts with its specific receptors, H_1 (Ash & Schild, 1966), H_2 (Black *et al.*, 1972) and H_3 (Arrang *et al.*, 1983), and with the polyamine-binding site on the NMDA receptor complex (Vorobjev *et al.*, 1993). The histamine-induced inhibition of K^+-evoked release of ACh from the cortex of freely moving rats is attributable to activation of H_3 receptors, as shown by the effects of histamine and the H_3 receptor selective agonists in moderating release, and by complete blockade of the inhibition by the H_3 receptor antagonist, clobenpropit. Histamine itself is active at concentrations consistent with those activating the H_3 receptor *in vitro* (Arrang *et al.*, 1983). Moreover, the highly selective H_3 receptor agonists, RAMH, imetit and immepip, mimic histamine-induced inhibition. Clobenpropit, a competitive antagonist at H_3 receptors with reported pA_2 values of 9.5 in mouse cortical slices (Kathmann *et al.*, 1993) and 9.9 in guinea-pig ileum (Van der Goot *et al.*, 1992), administered at nanomolar concentrations directly into the cortex, fully antagonizes the inhibition produced by 100 μM histamine. Confirmation of H_3 receptor involvement stems also from observations that histamine-induced inhibition is resistant to antagonism by triprolidine and cimetidine at concentrations more than 400 times their K_d for the H_1 (Ison *et al.*, 1971) and the H_2 (Durant *et al.*, 1977) receptor, respectively. Furthermore, histamine receptor agonists TEA and dimaprit, at concentrations sufficient to activate fully H_1 (Ganellin, 1982) and H_2 (Parson *et al.*, 1977) receptors, respectively, are completely inactive in modulating K^+-evoked release of ACh.

Recent reports show that RAMH inhibits 20 mM K^+-evoked release of [^{3}H]-ACh from slices of enthorinal cortex preloaded with [^{3}H]-choline (Clapham & Kilpatrick, 1992; Arrang *et al.*, 1995) and the maximal inhibition, about 40%, was similar to that observed in this study. In analogy with observations on ACh release, RAMH inhibited K^+-evoked [^{3}H]-histamine release from cortical slices preloaded with [^{3}H]-histidine by approximately 50% (Arrang *et al.*, 1987a), but, in the [^{3}H]-noradrenaline (Schlicker *et al.*, 1989) and [^{3}H]-5-hydroxytriptamine (Schlicker *et al.*, 1988) release assays, the inhibitory effects of RAMH were considerably less, maximal inhibition being approximately 25%. In the cortical slice, a 10 fold increase of the potency of RAMH in inhibiting K^+-evoked release of [^{3}H]-ACh was observed in the presence of mepyramine and ranitidine, antagonists at H_1 and H_2 receptors (Clapham & Kilpatrick, 1992; Arrang *et al.*, 1995). However, the maximal inhibition remained unchanged (Clapham & Kilpatrick. 1992; Arrang *et al.*, 1995). *In vivo*, H_1 and H_2 receptor blockade increased significantly the inhibition of K^+-evoked ACh produced by 0.1 μM histamine, a concentration clearly submaximal (see Figure 2), but failed to potentiate the effect elicited by 1 μM histamine. However, at this concentration, histamine triggered an almost maximal inhibition (see Figure 2). The lack of effectiveness of H_1 and H_2 receptor agonists may preclude invoking either the H_1 or the H_2 receptor in promoting the release of ACh from the cortex, but, the complexity of the *in vivo* model might mask stimulatory roles for H_1 and H_2 receptors. Alternatively, H_1 and H_2 receptor agonists were ineffective, for the release triggered by 100 mM K^+ was already maximal. Stimulation of transmitter release by H_2 receptor activation occur in other brain regions, since H_2 receptor activation increases the re-

lease of ACh from the hippocampus of anaesthetized rats (Mochizuki *et al.*, 1994) and of endogenous noradrenaline from rat hypothalamic slices (Blandina *et al.*, 1989).

The concentration of K^+ used in this study is only apparently very high, considering the low recovery of the microdialysis, and the rapid dilution into the extracellular liquid of the K^+ which crossed the membrane. Indeed, 60 mM K^+ has only a slight effect on ACh output during brain dialysis (Westerink *et al.*, 1987), and exposure of cortical slices to 20 mM K^+ (Clapham & Kilpatrick, 1992) and of the cortices of freely moving rats to 100 mM K^+ elicited increases in ACh release of similar amplitude.

ACh release evoked by 100 mM K^+ does not show any significant TTX-sensitive component, thus excluding involvement of neuronal loops. Conversely, TTX decreases ACh spontaneous release, indicating the presence of spontaneous impulse activity either in cholinergic afferents or in axons impinging on them. The presence of TTX abolishes completely imetit-induced inhibition, strongly suggesting that H_3 receptors modulating ACh release are not located presynaptically on cholinergic nerve terminals, or on non-cholinergic nerve endings impinging on the former. This hypothesis is consistent with the failure of H_3 receptor agonists to alter the K^+-evoked release of [^{3}H]-ACh from synaptosomes of the enthorinal cortex (Arrang *et al.*, 1995), and with the decrease of H_3 receptors observed in the cerebral cortex and striatum after local infusion of neurotoxins (Cumming *et al.*, 1991; Pollard *et al.*, 1993). Hence, the inhibition is likely to be indirect, mediated by the release of another transmitter(s) which, in turn, inhibits ACh release. Substances present in the cortex and inhibiting ACh release are noradrenaline (Beani *et al.*, 1978), dopamine (De Belleroche *et al.*, 1982) and adenosine (Vizi & Knoll, 1976). The potential candidate is probably released by cortical interneurones.

A physiological function of H_3 receptors in the control of ACh release is suggested by the facilitatory effect of thioperamide observed both in rat cortical slices (Clapham & Kilpatrick, 1992) and in the hippocampus of anaesthetized rats (Mochizuki *et al.*, 1994). However, clobenpropit alone failed to modify the release of ACh evoked by 100 mM K^+ from the cortex of freely moving rats. This is not surprising, since, under these conditions, the release of ACh evoked by 100 mM K^+ failed to show any significant TTX-sensitive component, thus excluding involvement of neuronal loops. Furthermore, the observation that thioperamide improved cognitive performances in senescence-accelerated but not in normal-rate ageing mice (Meguro *et al.*, 1995) suggests that the participation of H_3 receptors in the tonic modulation of cortical ACh release may become critical only under memory-impairing conditions.

Histaminergic neurones constitute a single neuronal group, localized in the tuberomammilary nucleus, with diffuse varicose projections to many brain areas (Panula *et al.*, 1989; Watanabe *et al.*, 1984). Consistent with this wide-ranging output, histamine may contribute to several functions such as arousal, locomotor activity, analgesia, thermoregulation, feeding and drinking (Prell *et al.*, 1986; Hough, 1988; Schwartz *et al.*, 1991). Recent work suggests an involvement of the histaminergic system in cognitive functions (De Almeida *et al.*, 1989; Cacabelos & Alvarez, 1991; Kamei & Tasaka, 1991), but the mechanism is unknown. Object recognition and passive avoidance response may involve the cortical cholinergic system, since both tasks involve a form of memory (short term/working memory) known to depend on the intact frontal cortex (Goldman-Rakic, 1987; Petrides, 1994), and are impaired by cholinoceptor antagonists (Spignoli & Pepeu, 1987; Ennaceur & Meliani, 1992). If cognitive deficits are related to reduced availability of ACh in the synaptic cleft (Quirion *et al.*, 1995), and H_3 receptor activation reduces ACh release, H_3 receptor activation would be expected to impair learning and memory. The present study confirms this prediction: systemic administration of doses of RAMH and imetit that reduced K^+-evoked cortical ACh release also impaired rat performance in object recognition and a passive avoidance response. Animals receiving imetit or RAMH did not differ from controls in the training trial of the passive avoidance response, or in the first trial of the object recognition, suggesting that the drugs do not alter motor or exploratory activity. This is consistent with studies in the mouse, which failed to show any effect of RAMH, 20 (Clapham & Kilpatrick, 1994) or 50 mg kg^{-1} i.p. (Sakai *et al.*, 1993) on locomotor activity. In the present study, H_3 receptor agonists were administered before training, so histamine H_3 receptor activation may act by suppressing acquisition. Interestingly, bilateral lesions of the tuberomammilary nucleus produce facilitation of learning (Klapdor *et al.*, 1994). In contrast to the hypothesis that histamine impairs cognitive functions, other studies suggest the positive role of the histaminergic system on learning and memory (Kamei *et al.*, 1993), and of RAMH in enhancing spatial learning in a water maze test (Smith *et al.*, 1994). Differences among the behavioural tests explain the discrepancies. Indeed, spatial learning is a primary function of the rodent hippocampus (O'Keefe & Nadel, 1978), and water maze test (Morris, 1981) is exquisitely sensitive to hippocampal lesions (Morris *et al.*, 1982; Sutherland *et al.*, 1983). Also the observation that histamine excited cholinergic nucleus basalis neurones by activation of H_1 and H_2, but not H_3 receptors (Khateb *et al.*, 1995), helps resolve the controversy concerning the role of histamine and the effects of drugs acting on histamine receptors on cognitive processes (Miyazaki *et al.*, 1995).

Uptake of circulating choline into the brain is reduced in older adults (Cohen *et al.*, 1995), and may be a contributing factor to late life onset of neurodegenerative, particularly dementing, illnesses in which cholinergic neurones show especial susceptibility to loss. Brain histaminergic activity is higher in the elderly (Prell *et al.*, 1988; 1991), and histamine content of the rat brain increases with age (Onodera *et al.*, 1992). These observations could be readily integrated: decreased choline uptake in the elderly may be due to feed-back inhibition produced by decreased ACh release, to which high histaminergic activity may be contributing. Treatment strategies could take advantage of non-cholinergic drugs that potentiate cholinergic functions. H_3 receptor antagonists could relieve histamine inhibitory effects and hence facilitate ACh release. The implications of the present study and the report that the H_3 receptor antagonist, thioperamide, improves learning and memory in senescence-accelerated (Meguro *et al.*, 1995) and scopolamine-impaired (Miyazaki *et al.*, 1995) mice support this contention.

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