British Journal of Pharmacology (2001) 132, 1665–1672

www.nature.com/bjp

Novel histamine H_3-receptor antagonists and partial agonists with a non-aminergic structure

[1]Tobias Nickel, [1]Ulrich Bauer, *,[1]Eberhard Schlicker, [1]Markus Kathmann, [1]Manfred Göthert, [2]Astrid Sasse, [2]Holger Stark & [2]Walter Schunack

[1]Institut für Pharmakologie und Toxikologie, Universität Bonn, Reuterstr. 2b, 53113 Bonn, Germany and [2]Institut für Pharmazie, Freie Universität Berlin, Königin-Luise-Str. 2+4, 14195 Berlin, Germany

1 We determined the affinities of eight novel histamine H_3-receptor ligands (ethers and carbamates) for H_3-receptor binding sites and their agonistic/antagonistic effects in two functional H_3-receptor models. The compounds differ from histamine in that the ethylamine chain is replaced by a propyloxy chain; in the three ethers mentioned below (FUB 335, 373 and 407), *R* is *n*-pentyl, 3-methylbutyl and 3,3-dimethylbutyl, respectively.

2 The compounds monophasically inhibited [^{3}H]-N^α-methylhistamine binding to mouse cerebral cortex membranes (pK_i 7.51–9.53).

3 The concentration-response curve of histamine for its inhibitory effect on the electrically evoked [^{3}H]-noradrenaline overflow from mouse cortex slices was shifted to the right by these compounds (apparent pA_2 6.61–8.00). Only FUB 373 and 407 inhibited the evoked overflow by themselves (intrinsic activities 0.3 and 0.4); these effects were counteracted by the H_3-receptor antagonist clobenpropit.

4 [^{35}S]-GTPγS binding to mouse cortex membranes was stimulated by the H_3-receptor agonist (*R*)-α-methylhistamine in a manner sensitive to clobenpropit. Among the novel compounds only FUB 373 and 407 stimulated [^{35}S]-GTPγS binding (intrinsic activities 0.6 and 0.4).

5 In conclusion, the novel compounds are partial H_3-receptor agonists (FUB 373 and 407) or H_3-receptor antagonists; comparison with FUB 335 shows that the transition from antagonist to agonist is caused by a slight structural change. A protonated N atom in the side chain is not necessary for agonism at H_3 receptors, proposing a receptor-ligand interaction different from that of classical agonists.

British Journal of Pharmacology (2001) **132,** 1665–1672

Keywords: H_3 receptors; [^{35}S]-GTPγS binding; [^{3}H]-N^α-methylhistamine binding; noradrenaline release; mouse brain cortex; partial agonists; histamine; (*R*)-α-methylhistamine; clobenpropit; presynaptic receptors

Abbreviations: Asp, aspartate; Clob., clobenpropit; [^{35}S]-GTPγS, guanosine 5′-[γ-^{35}S]-thiotriphosphate; PSS, physiological salt solution; Rα-MH, (*R*)-α-methylhistamine; S, electrical stimulation; spec. act., specific activity; t, collection period in which basal tritium efflux was determined

Introduction

The histamine H_3 receptor, which belongs to the superfamily of G protein-coupled receptors and has been cloned recently (Lovenberg *et al*., 1999; 2000; Tardivel-Lacombe *et al*., 2000), was discovered on histaminergic neurones as a presynaptic autoreceptor reducing the exocytotic release and the synthesis of histamine (Arrang *et al*., 1983; 1987). This receptor, however, also occurs as a presynaptic heteroreceptor on non-histaminergic nerve endings and inhibits the release of the respective transmitters (for review, see Stark *et al*., 1996; Hill *et al*., 1997). In addition, H_3 receptors have been found on several types of paracrine cells where they modulate the release of the respective mediator (for review, see Stark *et al*., 1996; Hill *et al*., 1997). Finally, a few papers suggest that H_3 receptors are also capable of inhibiting carrier-mediated noradrenaline release in the heart (for review, see Levi & Smith, 2000). A series of indications has been considered for H_3-receptor ligands; e.g., H_3-receptor antagonists penetrating the blood-brain barrier have been proposed as potential drugs for the treatment of dementia, narcolepsy and obesity (Stark *et al*., 1996; Leurs *et al*., 1998). One H_3-receptor antagonist, GT-2331 (Perceptin^R), is currently being evaluated in a phase II clinical study as a treatment for attention-deficit hyperactivity disorder (for review, see Tozer & Kalindjian, 2000).

The monoamines and histamine contain, in their side chain, an amino nitrogen which is protonated under physiological conditions. It is assumed that this protonated nitrogen forms an ionic bond with a (highly conserved) aspartate (Asp) residue in the third transmembrane domain of the respective receptor protein (for review, see Birdsall, 1991). Such an aspartate residue also occurs in the sequence of the recently cloned human (Asp-114), rat (Asp-114) and guinea-pig H_3 receptor (Asp-115) and may bind to the protonated side chain nitrogen of histamine and H_3-receptor agonists (for discussion, see Leurs *et al*., 2000). This hypothesis is based on several molecular modelling studies (Sippl *et al*., 1995; 1998; de Esch *et al*., 2000). On the other

*Author for correspondence; E-mail: e.schlicker@uni-bonn.de

hand, some congeners of histamine in which the ethylamine side chain is replaced by a side chain not positively charged at physiological pH proved to be partial or even full agonists, including ethers (Schlicker *et al*., 1996; Watt *et al*., 1997; Sasse *et al*., 1999; Lovenberg *et al*., 2000) and carbamates (Sasse *et al*., 1999; 2000).

It was the aim of the present study to determine the affinities and potencies of four recently synthesized ethers and four carbamates (Sasse *et al*., 1999 and unpublished) at the H_3 receptor in the mouse brain cortex. Affinities were determined using the radioligand [^{3}H]-N^α-methylhistamine. Potencies and intrinsic activities were determined using stimulation of guanosine 5′-[γ-^{35}S]-thiotriphosphate ([^{35}S]-GTPγS) binding and inhibition of noradrenaline release.

Methods

Binding studies

Cerebral cortex membranes from male NMRI mice or male Wistar rats were homogenized (Potter-Elvehjem) in 25 volumes of ice-cold Tris-HCl buffer (Tris 50 mM, pH 7.5; EDTA 5 mM; sucrose 10.27%) and centrifuged at $1000 \times g$ for 10 min (4°C). The supernatant was centrifuged at $35{,}000 \times g$ for 10 min and the pellet was resuspended in buffer and frozen at −80°C. The buffer was composed as follows (mM): Tris 50, pH 7.5; EDTA 5 for binding experiments with [^{3}H]-N^α-methylhistamine and Tris 50, pH 7.4; EGTA 0.2; $MgCl_2$ 3; NaCl 100 for binding experiments with [^{35}S]-GTPγS.

Binding experiments with [^{3}H]-N^α-methylhistamine were performed essentially as described by Kathmann *et al*. (1993). Briefly, membranes were incubated with Tris-HCl buffer in a final volume of 0.5 ml containing 30–60 μg protein. [^{3}H]-N^α-Methylhistamine was used at seven concentrations for saturation experiments and at a concentration of 0.5 nM for competition experiments. The incubation (30°C) was terminated after 40 min by filtration through polyethyleneimine (0.3%)-pretreated Whatman GF/C filters. (*R*)-α-Methylhistamine 2 μM was used to determine non-specific binding (15% for [^{3}H]-N^α-methylhistamine 0.5 nM). Protein concentration was assayed by the method described by Bradford (1976). Data were analysed using the program GraphPadPrism (Prism; GraphPad Software, San Diego, CA, U.S.A.).

Binding experiments with [^{35}S]-GTPγS (carried out essentially as described by Nakazi *et al*. 2000) were performed in Tris-HCl buffer (mM: Tris 50, pH 7.4; EGTA 0.2; $MgCl_2$ 3; NaCl 100; GDP 30 μM) in a final volume of 0.5 ml containing 5–15 μg protein. [^{35}S]-GTPγS was used at a concentration of 0.05 nM. The incubation (30°C) was terminated after 60 min by filtration through Whatman GF/B filters. Non-radioactive guanosine 5′-O-(3-thiotriphosphate) 10 μM was used to determine non-specific binding (20% of basal binding).

Superfusion studies

Cerebral cortex slices (0.3 mm thick, 3 mm diameter) from male NMRI mice were incubated (37°C) for 60 min with physiological salt solution (PSS) containing [^{3}H]-noradrenaline 0.025 μM. Subsequently, the slices were transferred to superfusion chambers and superfused (0.5 ml min^{-1}) with PSS (37°C) containing desipramine 1 μM (for blockade of the neuronal noradrenaline transporter) and rauwolscine 1 μM (for blockade of α_2-adrenoceptors). The superfusate was collected in 5-min samples; experiments lasted for 110 min. Tritium overflow was evoked by two 2-min periods of electrical field stimulation (0.3 Hz, 50 mA, 2 ms) after 40 and 90 min of superfusion (S_1 and S_2). The drugs under study were present in the medium either throughout superfusion or from 62 min of superfusion onward, as indicated under Results. For composition of the PSS, see Werthwein *et al*. (1999).

Tritium efflux was calculated as the fraction of the tritium content in the slices at the beginning of the respective collection period (fractional rate of tritium efflux). To quantify effects of drugs on basal efflux, the ratio of the fractional rates in the 5 min period prior to S_2 (t_2) and in the 5 min period 15–20 min after the onset of S_1 (t_1) was determined (for drugs added to the PSS from 62 min of superfusion onward) or the t_1 values obtained in the absence or presence of a given drug were directly compared to each other (for drugs present in the PSS throughout superfusion). Stimulation-evoked tritium overflow was calculated by subtraction of basal from total efflux during stimulation and the subsequent 13 min and expressed as per cent of the tritium present in the slice at the onset of stimulation (basal efflux was assumed to decline linearly from the 5 min period before to that 15–20 min after onset of stimulation). To quantify drug-induced effects on the stimulated tritium overflow, the ratio of the overflow evoked by S_2 over that evoked by S_1 was determined (S_2/S_1) (for drugs added to the PSS from 62 min of superfusion) or the S_1 values obtained in the absence or presence of a given drug were directly compared to each other (for drugs present throughout superfusion). Apparent pA_2 values were calculated according to formula 4 of Furchgott (1972).

Statistics

Results are given as means ± s.e.mean of *n* experiments (superfusion) and of *n* experiments in triplicate (binding). Student's *t*-test was used for comparison of mean values; the Bonferroni correction was used when two or more values had to be compared to the same control. The *F*-test was applied in order to evaluate whether the inhibition of [^{3}H]-N^α-methylhistamine binding by drugs is better fitted by a one- or a two-site model.

Drugs used

(*R*)-(−)-[Ring-2,5,6-^{3}H]-noradrenaline (specific activity (spec. act.) 51.8–62.3 Ci mmol^{-1}; NEN, Zaventem, Belgium); [methyl-^{3}H]-N^α-methylhistamine dihydrochloride (spec. act. 78.9 Ci mmol^{-1}; NEN); [^{35}S]-GTPγS, base (spec. act. 1250 Ci mmol^{-1}; NEN) or triethylammonium salt (spec. act. 1099-1105 Ci mmol^{-1}; Amersham, Braunschweig, Germany); clobenpropit dihydrobromide (Professor H. Timmerman, Vrije Universiteit Amsterdam, The Netherlands); desipramine hydrochloride (Ciba-Geigy, Wehr, Germany); histamine dihydrochloride (Sigma, Munich, Germany); the test compounds FUB 335, FUB 373, FUB 379, FUB 397, FUB 407, FUB 415, FUB 474 and FUB 475 (maleates; for chemical structure, see Table 1) were synthesized at the

Institut für Pharmazie (Freie Universität Berlin, Germany; Sasse *et al*., 1999 and unpublished); (*R*)-α-methylhistamine dihydrogenmaleate (Institut für Pharmazie, Freie Universität Berlin); rauwolscine hydrochloride (Roth, Karlsruhe, Germany). Stock solutions of the drugs were prepared with water and diluted with PSS (superfusion experiments) or water (binding experiments) to the concentration required.

Results

Inhibition of [^{3}H]-N^α-methylhistamine binding

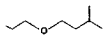
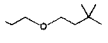
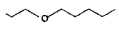
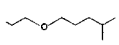
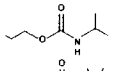
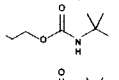
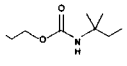
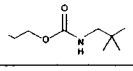
In saturation binding experiments on mouse brain cortex membranes, using [^{3}H]-N^α-methylhistamine at seven concentrations, a K_D value of 0.71 ± 0.08 nM with a maximum number of binding sites (B_{max}) of 111 ± 2 fmol mg^{-1} protein was determined; Scatchard analysis revealed a straight line with a Hill coefficient (n_H) of unity (Figure 1). Similar results had been obtained previously (Kathmann *et al*., 1993) for saturation binding experiments with this radioligand on rat brain cortex membranes. In those experiments, the K_D was 0.70 ± 0.03 nM and the B_{max} amounted to 98 ± 6 fmol mg^{-1} protein.

In competition binding experiments, binding of [^{3}H]-N^α-methylhistamine 0.5 nM to mouse cortex membranes was inhibited monophasically (n_H near unity) by the eight FUB compounds yielding pK_i values ranging from 7.51 to 9.53 (Figure 2, Table 1). When the compounds were studied in rat brain cortex membranes, very similar results were obtained (Table 1; highly significant ($P<0.001$) correlation of the pK_i values in membranes from both species with a correlation coefficient r of 0.978; regression line not shown).

Stimulation of [^{35}S]-GTPγS binding

Basal [^{35}S]-GTPγS binding to mouse brain cortex membranes was 153 ± 49 fmol mg^{-1} protein. (*R*)-α-Methylhistamine, which is a full agonist with respect to H_3 receptor-mediated stimulation of [^{35}S]-GTPγS binding in cerebral cortex membranes from the rat (Clark & Hill, 1996) and the mouse (own unpublished results), stimulated [^{35}S]-GTPγS binding to mouse cortex membranes by about 20% (Figure 3). The

Table 1 Chemical structures, affinities, potencies and intrinsic activities (i.a.) of newly synthesized H_3-receptor ligands in the rat and mouse brain cortex

		Rat	*Mouse*	*Mouse*		*Mouse*	
		Inhibition of [^{3}H]-N^α-methyl-histamine binding	Inhibition of [^{3}H]-N^α-methyl-histamine binding	Stimulation of [^{35}S]-GTPγS binding		Effect on H_3 receptor inhibiting noradrenaline release	
Compound	*R*	*pK$_i$*[1]	*pK$_i$*[2]	*pEC$_{50}$*[3]	*i.a.*[3]	*apparent pA$_2$*[4]	*i.a.*[5]
(*R*)-α-Methyl-histamine		-[6]	-	7.77	1.0	-	-
Histamine		-	-	-	-	-	1.0
FUB 373		8.88	8.91	7.76	0.6	7.41	0.3
FUB 407		9.42	9.51	7.99	0.4	7.61	0.4
FUB 335		8.62	8.72	-	0	8.00	0
FUB 397		9.23	9.53	-	0	7.60	0
FUB 415		7.13	7.51	-	0	7.07	0
FUB 379		7.95	7.84	-	0	6.61	0
FUB 474		8.47	8.60	-	0	7.41	0
FUB 475		8.54	8.68	-	0	7.48	0

[1] Determined from concentration-response curves similar to those in Figure 2 (not shown); n=4, s.e.m. values ranged from 0.01 to 0.24.
[2] Determined from the concentration-response curves shown in Figure 2; n=4, s.e.m. values ranged from 0.04 to 0.13.
[3] Determined from the concentration-response curves shown in Figure 3 ((*R*)-α-methylhistamine, FUB 373 and FUB 407; note that the other FUB compounds did not significantly stimulate [^{35}S]-GTPγS binding; not shown); n=16.
[4] Determined from the concentration-response curve shown in Figure 4 (FUB 335) or from similar curves not shown (other FUB compounds); n=4-11, s.e.m. values ranged from 0.06 to 0.17. The following concentrations of the FUB compounds were used: FUB 373 0.32 μM; FUB 407 0.32 μM; FUB 335 0.1 μM; FUB 397 0.32 μM; FUB 415 3.2 μM; FUB 379 3.2 μM; FUB 474 0.32 μM; FUB 475 0.32 μM.
[5] Determined from the results shown in Figure 5 (histamine, FUB 373 and FUB 407; note that the other FUB substances did not significantly inhibit noradrenaline release; not shown), n=8. The following concentrations of the FUB compounds were used (100×10^{-pA_2}): FUB 373 3.9 μM; FUB 407 2.5 μM; FUB 335 1 μM; FUB 397 2.5 μM; FUB 415 8.5 μM; FUB 379 25 μM; FUB 474 3.9 μM; FUB 475 3.3 μM.
[6] The symbol "-" either means "not determined" or "not applicable".

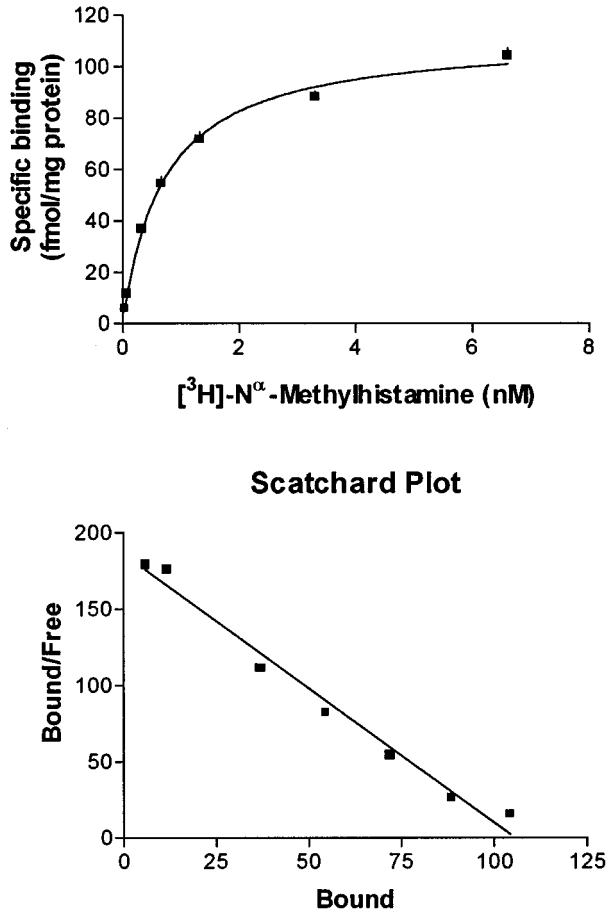


Figure 1 Saturation of specific [^{3}H]-N^α-methylhistamine binding to mouse brain cortex membranes. Membranes were incubated (30°C) for 40 min with [^{3}H]-N^α-methylhistamine. Specific binding was defined as that inhibited by (*R*)-α-methylhistamine 2 μM. Scatchard transformation of the saturation data is presented in the lower panel. Means from four experiments (in triplicate) are shown (for some data points, s.e.mean is contained within the symbol).

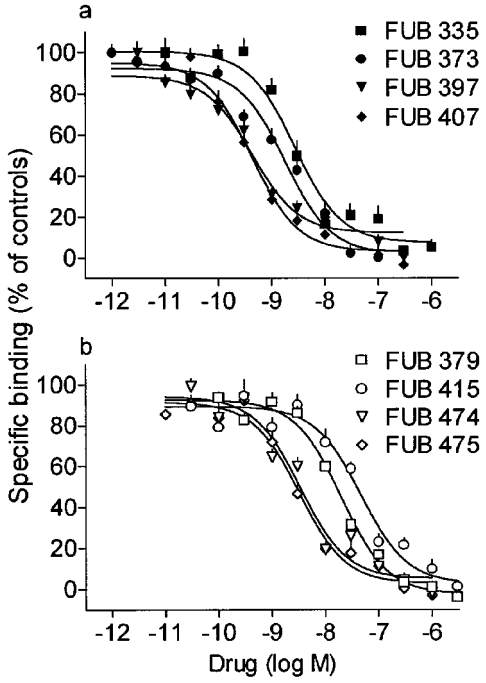


Figure 2 Inhibition of specific [^{3}H]-N^α-methylhistamine binding to mouse brain cortex membranes by eight novel H_3-receptor ligands with ether (a) or carbamate structure (b). Membranes were incubated (30°C) for 40 min with [^{3}H]-N^α-methylhistamine and 10 concentrations of the test compounds. Means±s.e.mean from four experiments (in triplicate) are shown (for some data points, s.e.mean is contained within the symbol).

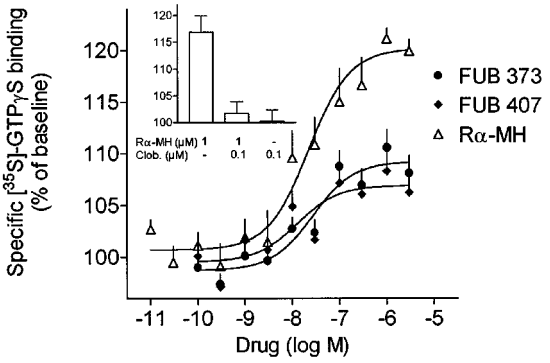


Figure 3 Effect of (*R*)-α-methylhistamine (Rα-MH) and of two novel H_3-receptor ligands on specific [^{35}S]-GTPγS binding to mouse brain cortex membranes. Membranes were incubated (30°C) for 60 min with [^{35}S]-GTPγS and 10 concentrations of the test compounds. Means±s.e.mean from 16 (FUB compounds) or seven experiments (Rα-MH) in triplicate (for some data points, s.e.mean is contained within the symbol). The *inset* shows the interaction of Rα-MH with clobenpropit (Clob.); means±s.e.mean from five experiments in triplicate.

effect of (*R*)-α-methylhistamine 1 μM (concentration producing the maximum effect; EC_{50} 0.02 μM) was abolished by the H_3-receptor antagonist clobenpropit 0.1 μM, which, by itself, did not affect binding (Figure 3, inset). Among the eight FUB compounds, only FUB 373 and FUB 407 stimulated [^{35}S]-GTPγS binding, behaving as partial agonists in this respect (Figure 3, Table 1).

Inhibition of [^{3}H]-noradrenaline release

Basal tritium efflux was not affected by histamine, the FUB compounds and clobenpropit (not shown). The electrically evoked tritium overflow (S_2/S_1) was 1.17±0.02 in 78 controls; tritium overflow evoked by S_1 amounted to 8.18±0.24% of tissue tritium ($n=78$). The electrically evoked tritium overflow was inhibited by histamine (added to the medium before S_2) in a concentration-dependent manner (Figure 4); the maximum extent of inhibition amounted to about 50% with an EC_{50} value of 0.19 μM. The concentration–response curve of histamine was shifted to the right by the FUB compounds (present in the medium during S_1 and S_2). The concentration–response curve for histamine obtained in the presence of FUB 335 is shown in Figure 4; similar curves were obtained for the other FUB compounds (not shown). The apparent pA_2 values, ranging from 6.61 to 8.00, are given in Table 1. FUB 373 0.32 μM, by itself, decreased the evoked tritium overflow (S_1) by 10±3% ($P<0.05$; $n=8$) and FUB 407 0.32 μM tended to decrease it (by 20±6%; $P=0.08$; $n=13$). The other FUB compounds did not affect the evoked overflow by themselves (not shown).

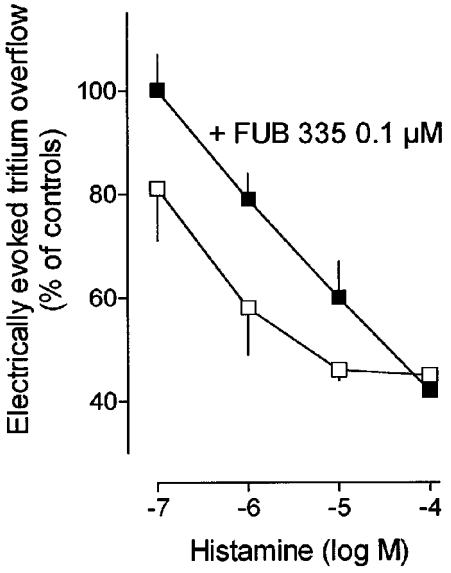


Figure 4 Effect of histamine on the electrically evoked tritium overflow from superfused mouse brain cortex slices preincubated with [^{3}H]-noradrenaline and interaction of the novel H_3-receptor ligand FUB 335 with histamine. The medium contained desipramine 1 μM, rauwolscine 1 μM and, when necessary, FUB 335 throughout superfusion (37°C, 110 min) and histamine from 62 min of superfusion onward. Tritium overflow was evoked twice, after 40 and 90 min of superfusion (S_1, S_2), and the ratio of the overflow evoked by S_2 over that evoked by S_1 was determined. Results are given as per cent of the tritium overflow (S_2/S_1) in controls (not shown). Means ± s.e.mean from 4–5 experiments are shown (for some data points, s.e.mean is contained within the symbol).

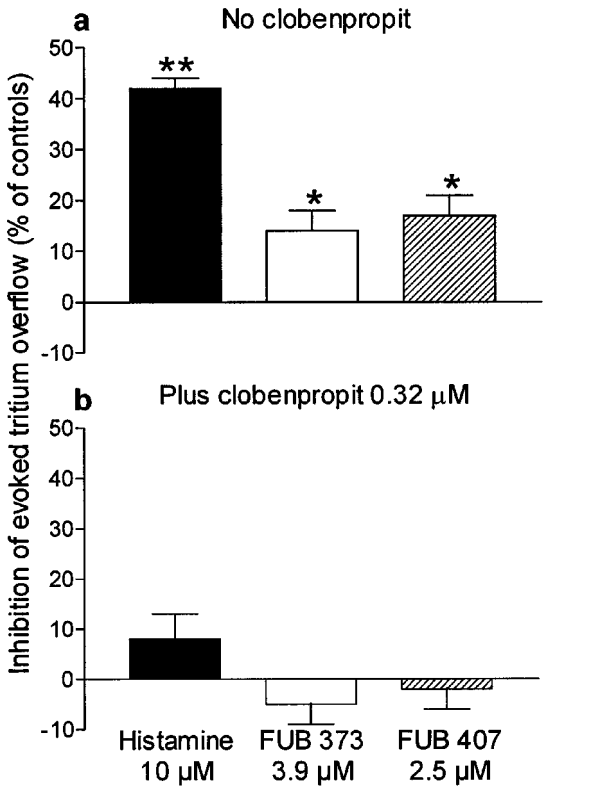


Figure 5 Inhibition of the electrically evoked tritium overflow (from superfused mouse brain cortex slices preincubated with [^{3}H]-noradrenaline) by histamine and two novel H_3-receptor ligands in the absence (a) or presence of clobenpropit (b). The medium contained desipramine 1 μM, rauwolscine 1 μM and, when necessary, clobenpropit throughout superfusion (37°C, 110 min) and histamine or the FUB compound from 62 min of superfusion onward. Tritium overflow was evoked twice, after 40 and 90 min of superfusion (S_1, S_2), and the ratio of the overflow evoked by S_2 over that evoked by S_1 was determined (S_2/S_1). Results are given as per cent of the tritium overflow (S_2/S_1) in controls and the inhibition of the evoked overflow (in per cent of controls) was determined according to the following formula: $100 \times (1 - S_2/S_1$ {in the presence of the test compound}$/S_2/S_1$ {in the absence of the test compound}). Means ± s.e.mean from 7–8 experiments. $^*P < 0.05$, $^{**}P < 0.001$, refers to the comparison of the S_2/S_1 value in the presence, to that obtained in the absence, of histamine or the FUB compound.

In order to further investigate potential inhibitory effects of the FUB compounds on the electrically evoked tritium overflow, the compounds were added to the medium before S_2 (like histamine in the experiments of Figure 4) and were studied at a concentration exceeding the respective antagonist dissociation constant (10^{-pA_2}) by a factor of 100. Histamine 10 μM was used as a reference drug. Among the FUB compounds, only FUB 373 and FUB 407 inhibited the electrically evoked tritium overflow (S_2/S_1), behaving as partial agonists in this respect (Figure 5a, Table 1). The inhibitory effects of the two FUB compounds and of histamine were counteracted by clobenpropit 0.32 μM (present during S_1 and S_2; Figure 5b), which, by itself, did not affect the evoked tritium overflow (S_1; not shown).

Discussion

The affinities/potencies of eight novel H_3-receptor ligands (four ethers and four carbamates; Sasse *et al*., 1999 and unpublished) have been determined at three subsequent levels of the signal transduction chain of the H_3-receptor in the mouse brain cortex, i.e. at the level (i) of the receptor, (ii) of the G proteins, and (iii) of an effector (H_3-receptor-mediated inhibition of noradrenaline release in cerebral cortex slices; Schlicker *et al*., 1992).

The eight compounds under study proved to be antagonists at the H_3 receptor producing inhibition of the electrically evoked tritium overflow (which represents quasi-physiological noradrenaline release; Schlicker *et al*., 1992), the most potent compound being the ether FUB 335 with an apparent pA_2 of eight. Similar antagonist potencies of these compounds were obtained in another two functional H_3-receptor models, i.e., the H_3 autoreceptor in rat brain cortex synaptosomes (difference in potency estimates compared to the pA_2 values of the present study $\leqslant 0.5$ log units) and the H_3 heteroreceptor in guinea-pig ileum segments (difference $\leqslant 0.6$ log units) (Sasse *et al*., 1999 and unpublished). One has to recall that in our release model two electrical stimuli (S_1 and S_2) were administered and one of the eight compounds was present throughout the superfusion experiments, whereas the agonist (histamine) was present during S_2 only. To quantify the effect of histamine, S_2/S_1 values were determined which offer the advantage that the variation of S_1 does not impair the result. On the other hand, comparison of S_1 values (in the absence and presence of antagonists) is not particularly suited to quantify effects of the antagonists on noradrenaline release. Nonetheless, our results showed that two ethers

Table 2 Intrinsic activities of newly synthesized H_3-receptor ligands in five functional H_3-receptor models and differences between their pK_i ([^{3}H]-N^α-methylhistamine binding) and apparent pA_2 values (H_3 receptor causing inhibition of noradrenaline release)

	Intrinsic activities[a]				*Differences between* pK_i[b] *and apparent* pA_2[c]
	Mouse brain cortex slices	*Mouse brain cortex membranes*	*Rat brain cortex synaptosomes*	*Mouse brain cortex* in vivo	
Compound	*Inhibition of noradrenaline release*[d]	*Stimulation of [^{35}S]-GTPγS binding*[d]	*Inhibition of histamine release*[e]	*Inhibition of formation of N^τ-methylhistamine*[e]	
FUB 373	0.3	0.6	0.2	1.0	1.50
FUB 407	0.4	0.4	0.6	1.0	1.90
FUB 335	0	0	0	–[f]	0.72
FUB 397	0	0	0.3	0.6	1.93
FUB 415	0	0	0	0	0.44
FUB 379	0	0	0	0.7	1.23
FUB 474	0	0	0	0.4	1.19
FUB 475	0	0	0.2	0.7	1.20

[a]The intrinsic activities were 0 for all compounds in guinea-pig ileum segments in which the H_3-receptor-mediated inhibition of the electrically induced twitch response (due to acetylcholine release) was determined (Sasse *et al.* 1999 and unpublished). [b]Inhibition of [^{3}H]-N^α-methylhistamine binding (column 4 of Table 1). [c]Effect on H_3 receptor causing inhibition of noradrenaline release (column 7 of Table 1). [d]From Table 1. [e]From Sasse *et al.* (1999) and unpublished. [f]Compound devoid of *in vivo* activity up to 30 mg kg^{-1}.

(FUB 373 and FUB 407) decreased/tended to decrease noradrenaline release, suggesting that these compounds are partial H_3-receptor agonists.

To study potential agonistic effects of FUB 373 and FUB 407 and of the other compounds more precisely, experiments were performed in which the H_3-receptor ligand under study was added to the superfusion medium after S_1 and its effect on the S_2/S_1 value was determined. Unfortunately, whole concentration–response curves could not be obtained. The reason may be that the s.e.mean values (up to 10% of the S_2/S_1 values in the controls; see Figure 4) are relatively high when compared to the maximum inhibitory effects of partial agonists (since the maximum inhibitory effect is 50% for the full agonist histamine, a partial agonist with an intrinsic activity of 0.5 will be expected to inhibit noradrenaline release by maximally 25%). In order to overcome this problem, we studied that concentration of each compound that exceeds the respective antagonist dissociation constant (known from the interaction experiments with histamine) by a factor of 100. This approach is based on two rules of receptor theory, namely that the affinity of partial agonists in terms of agonism and antagonism should be identical and that the maximum effect of an agonist is reached at a concentration exceeding the EC_{50} value by a factor of about 10 to 100. Using this approach we found that FUB 373 and FUB 407 inhibited noradrenaline release, whereas the other FUB compounds failed to do so. Interaction experiments with clobenpropit revealed that FUB 373 and FUB 407 are true H_3-receptor agonists and that these compounds are not acting on some other way to stimulate noradrenaline release.

As a second functional H_3-receptor model, stimulation of [^{35}S]-GTPγS binding was examined. Our experiments with the H_3-receptor agonist (*R*)-α-methylhistamine and the H_3-receptor antagonist clobenpropit demonstrate that H_3-receptor-mediated activation of [^{35}S]-GTPγS binding, previously shown in brain membranes from the rat (Clark & Hill, 1996; Laitinen & Jokinen, 1998) and the zebrafish (Peitsaro *et al.*, 2000), is detectable in membranes from the mouse brain cortex as well. Among the novel compounds, only FUB 373 and FUB 407 showed a stimulatory effect. Thus, the latter two compounds are partial agonists in both functional H_3-receptor models (although the intrinsic activities are somewhat different in the two models). Moreover, the agonistic potencies of both compounds in the [^{35}S]-GTPγS binding assay resembled their antagonistic potencies in the release model.

The eight compounds were also studied in another three functional H_3-receptor models (Sasse *et al.*, 1999 and unpublished), i.e., at H_3 receptors causing inhibition of (i) the twitch response in guinea-pig ileum segments, (ii) histamine release in rat brain cortex synaptosomes, and (iii) formation of the histamine metabolite N^τ-methylhistamine in the mouse brain *in vivo*. Table 2 shows that none of the compounds possessed intrinsic activity at the H_3 receptor in the guinea-pig ileum, whereas six of the eight compounds showed an agonistic effect at the H_3 receptor *in vivo* (note that the situation for FUB 335 is unclear since this drug is inactive *in vivo* at doses up to 30 mg kg^{-1}; unpublished). The H_3 receptor in rat brain synaptosomes resembles the H_3 receptors examined in our study with respect to the intrinsic activities; the reason for the lack of agonism of FUB 397 and FUB 475 in our models might be that low intrinsic activities ($\leqslant 0.3$) escaped detection. The marked discrepancies of intrinsic activities in the five models shown in Table 2 may be explained by assuming that the degree of receptor coupling is low for the H_3 receptor in the guinea-pig ileum, moderate for the three H_3 receptors in rodent brain *in vitro*, and high for the H_3 receptor in the mouse brain *in vivo*. (Although the latter explanation may be the most likely one, the possibility that species differences (Lovenberg *et al.*, 2000) or splice variants (Tardivel-Lacombe *et al.*, 2000; Drutel *et al.*, 2001) contribute to the discrepancies cannot be excluded.) Further evidence for a marked difference in receptor coupling between the H_3 receptors in the guinea-pig small intestine and the mouse brain *in vitro* comes from experiments with the higher homologues of histamine (Leurs *et al.*, 1996). Thus, the *n*-propyl, *n*-butyl and *n*-pentyl homologues of histamine proved to be partial agonists at the H_3 receptor in the mouse brain cortex, but pure antagonists at the H_3 receptor in the guinea-pig jejunum.

The binding of the ligands to the H_3 receptor has been determined using [^{3}H]-N^α-methylhistamine. The B_{max} and K_D values obtained in saturation binding studies in mouse cortex membranes were very similar to those obtained in studies on rat cortex membranes, which were used in our previous studies (Schlicker *et al*., 1994; 1996; Leurs *et al*., 1996; Kathmann *et al*., 1998). Like in the study of Schlicker *et al*. (1994), the pK_i values obtained from the binding experiments with [^{3}H]-N^α-methylhistamine were statistically compared with the apparent pA_2 values from the superfusion experiments. Although a significant correlation was found for the H_3-receptor ligands examined by Schlicker *et al*. (1994), this did not hold true for the ligands considered in the present study, regardless of whether the pA_2 values were compared with the pK_i values obtained for binding experiments in the cerebral cortex from the rat ($r=0.62$) or the mouse ($r=0.69$). Table 2 shows that the difference between pK_i and pA_2 is 0.4 for FUB 415, which is devoid of intrinsic activity at any H_3 receptor; the small difference between both parameters may be explained by the fact that pA_2 values for antagonists are frequently somewhat smaller than their pK_i values (Schlicker *et al*., 1994; 1996; Kathmann *et al*., 1998). The difference between pK_i and pA_2 is between 1.2 and 1.9 for those six compounds that are (partial) agonists at the H_3 receptor in the mouse brain *in vivo*, the H_3-receptor model with the highest receptor coupling. The relatively high pK_i values of the latter six compounds can be explained by the fact that, with respect to G protein-coupled receptors, pK_i values of agonists are frequently particularly high when an agonist radioligand is used. The explanation for this phenomenon is that a ternary complex consisting of agonist radioligand, receptor and G protein is formed; under this experimental condition, the equilibrium dissociation constants are underestimated, i.e., relatively high affinities are obtained (Kenakin, 1993). Thus, not only the functional models examined in the present study but also the radioligand binding experiments with [^{3}H]-N^α-methylhistamine suggest that some of the ligands including FUB 373 and FUB 407 exhibit agonistic effects at H_3 receptors.

A comparison of the structures of FUB 335 and FUB 397 (which are devoid of an agonistic effect at the H_3 receptor causing noradrenaline release) and of FUB 373 and FUB 407 (which have a partial agonistic effect) reveals that already slight structural changes cause the transition from pure antagonism to partial agonism; steric effects rather than changes in lipophilicity are important in this respect. The results of the present and of some previous studies (Schlicker *et al*., 1996; Watt *et al*., 1997; Sasse *et al*., 1999; 2000; Lovenberg *et al*., 2000) show that a nitrogen (undergoing protonation at physiological pH) in the side chain is not a prerequisite for activation of the H_3-receptor.

This study was supported by a grant from the Deutsche Forschungsgemeinschaft (Bonn, Germany; Graduiertenkolleg 246 to E. Schlicker and M. Göthert) and by the Verband der Chemischen Industrie, Fonds der Chemie (Frankfurt/Main, Germany). We wish to thank Mrs D. Petri for her skilled technical assistance and Professor H. Timmerman and Ciba-Geigy for gifts of drugs.

References

ARRANG, J.-M., GARBARG, M. & SCHWARTZ, J.-C. (1983). Auto-inhibition of brain histamine release mediated by a novel class (H_3) of histamine receptor. *Nature,* **302,** 832–837.

ARRANG, J.-M., GARBARG, M. & SCHWARTZ, J.-C. (1987). Auto-inhibition of histamine synthesis mediated by presynaptic H_3-receptors. *Neuroscience,* **23,** 149–157.

BIRDSALL, N.J.M. (1991). Cloning and structure-function of the H_2 histamine receptor. *Trends Pharmacol. Sci.,* **12,** 9–10.

BRADFORD, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.,* **72,** 248–254.

CLARK, E.A. & HILL, S.J. (1996). Sensitivity of histamine H_3 receptor agonist-stimulated ^{35}S-GTPγS binding to pertussis toxin. *Eur. J. Pharmacol.,* **296,** 223–225.

DE ESCH, I.J.P., TIMMERMAN, H., MENGE, W.M.P.B. & NEDERKOORN, P.H.J. (2000). A qualitative model for the histamine H_3 receptor explaining agonistic and antagonistic activity simultaneously. *Arch. Pharm.,* **333,** 254–260.

DRUTEL, G., PEITSARO, N., KARLSTEDT, K., WIELAND, K., SMIT, M.J., TIMMERMAN, H., PANULA, P. & LEURS, R. (2001). Identification of rat H_3 receptor isoforms with different brain expression and signaling properties. *Mol. Pharmacol.,* **59,** 1–8.

FURCHGOTT, R.F. (1972). The classification of adrenoceptors (adrenergic receptors). An evaluation from the standpoint of receptor theory. In *Handbook of Experimental Pharmacology*, vol. XXXIII. ed. Blaschko, H. & Muscholl, E. pp. 283–335. Berlin: Springer.

HILL, S.J., GANELLIN, C.R., TIMMERMAN, H., SCHWARTZ, J.-C., SHANKLEY, N.P., YOUNG, J.M., SCHUNACK, W., LEVI, R. & HAAS, H.L. (1997). International Union of Pharmacology. XIII. Classification of histamine receptors. *Pharmacol. Rev.,* **49,** 253–278.

KATHMANN, M., SCHLICKER, E., DETZNER, M. & TIMMERMAN, H. (1993). Nordimaprit, homodimaprit, clobenpropit and imetit: affinities for H_3 binding sites and potencies in a functional H_3 receptor model. *Naunyn-Schmiedeberg's Arch. Pharmacol.,* **348,** 498–503.

KATHMANN, M., SCHLICKER, E., MARR, I., WERTHWEIN, S., STARK, H. & SCHUNACK, W. (1998). Ciproxifan and chemically related compounds are highly potent and selective histamine H_3-receptor antagonists. *Naunyn-Schmiedeberg's Arch. Pharmacol.,* **358,** 623–627.

KENAKIN, T. (1993). *Pharmacological analysis of drug-receptor interaction*. New York: Raven.

LAITINEN, J.T. & JOKINEN, M. (1998). Guanosine 5′-(γ-[^{35}S]thio)-triphosphate autoradiography allows selective detection of histamine H_3 receptor-dependent G protein activation in rat brain tissue sections. *J. Neurochem.,* **71,** 808–816.

LEURS, R., BLANDINA, P., TEDFORD, C. & TIMMERMAN, H. (1998). Therapeutic potential of histamine H_3 receptor agonists and antagonists. *Trends Pharmacol. Sci.,* **19,** 177–183.

LEURS, R., HOFFMANN, M., WIELAND, K. & TIMMERMAN, H. (2000). H_3 receptor gene is cloned at last. *Trends Pharmacol. Sci.,* **21,** 11–12.

LEURS, R., KATHMANN, M., VOLLINGA, R.C., MENGE, W.M.P.B., SCHLICKER, E. & TIMMERMAN, H. (1996). Histamine homologues discriminating between two functional H_3 receptor assays. Evidence for H_3 receptor heterogeneity. *J. Pharmacol. Exp. Ther.,* **276,** 1009–1015.

LEVI, R. & SMITH, N.C.E. (2000). Histamine H_3-receptors: a new frontier in myocardial ischemia. *J. Pharmacol. Exp. Ther.,* **292,** 825–830.

LOVENBERG, T.W., PYATI, J., CHANG, H., WILSON, S.J. & ERLANDER, M.G. (2000). Cloning of rat histamine H_3 receptor reveals distinct species pharmacological profiles. *J. Pharmacol. Exp. Ther.,* **293,** 771–778.

LOVENBERG, T.W., ROLAND, B.L., WILSON, S.J., JIANG, X., PYATI, J., HUVAR, A., JACKSON, M.R. & ERLANDER, M.G. (1999). Cloning and functional expression of the human histamine H_3 receptor. *Mol. Pharmacol.,* **55,** 1101–1107.

NAKAZI, M., BAUER, U., NICKEL, T., KATHMANN, M. & SCHLICKER, E. (2000). Inhibition of serotonin release in the mouse brain via cannabinoid CB_1 receptors. *Naunyn-Schmiedeberg's Arch. Pharmacol.,* **361,** 19–24.

PEITSARO, N., ANICHTCHIK, O.V. & PANULA, P. (2000). Identification of a histamine H_3-like receptor in the zebrafish (Danio rerio) brain. *J. Neurochem.,* **75,** 718–724.

SASSE, A., STARK, H., LIGNEAU, X., ELZ, S., REIDEMEISTER, S., GANELLIN, C.R., SCHWARTZ, J.-C. & SCHUNACK, W. (2000). (Partial) agonist/antagonist properties of novel diarylalkyl carbamates on histamine H_3 receptors. *Bioorg. Med. Chem.,* **8,** 1139–1149.

SASSE, A., STARK, H., REIDEMEISTER, S., HÜLS, A., ELZ, S., LIGNEAU, X., GANELLIN, C.R., SCHWARTZ, J.-C. & SCHUNACK, W. (1999). Novel partial agonists for the histamine H_3 receptor with high in vitro and in vivo activity. *J. Med. Chem.,* **42,** 4269–4274.

SCHLICKER, E., BEHLING, A., LÜMMEN, G. & GÖTHERT, M. (1992). Histamine H_{3A} receptor-mediated inhibition of noradrenaline release in the mouse brain cortex. *Naunyn-Schmiedeberg's Arch. Pharmacol.,* **345,** 489–493.

SCHLICKER, E., KATHMANN, M., BITSCHNAU, H., MARR, I., REIDEMEISTER, S., STARK, H. & SCHUNACK, W. (1996). Potencies of antagonists chemically related to iodoproxyfan at histamine H_3 receptors in mouse brain cortex and guinea-pig ileum: evidence for H_3 receptor heterogeneity? *Naunyn-Schmiedeberg's Arch. Pharmacol.,* **353,** 482–488.

SCHLICKER, E., KATHMANN, M., REIDEMEISTER, S., STARK, H. & SCHUNACK, W. (1994). Novel histamine H_3 receptor antagonists: affinities in an H_3 receptor binding assay and potencies in two functional H_3 receptor models. *Br. J. Pharmacol.,* **112,** 1043-1048 and 113, 657 (erratum).

SIPPL, W., STARK, H. & HÖLTJE, H.-D. (1995). Computer-assisted analysis of histamine H_2- and H_3-receptor agonists. *Quant. Struct. Act. Relat.,* **14,** 121–125.

SIPPL, W., STARK, H. & HÖLTJE, H.-D. (1998). Development of a binding site model for histamine H_3-receptor agonists. *Pharmazie,* **53,** 433–437.

STARK, H., SCHLICKER, E. & SCHUNACK, W. (1996). Developments of histamine H_3-receptor antagonists. *Drugs Future,* **21,** 507–520.

TARDIVEL-LACOMBE, J., ROULEAU, A., HERON, A., MORISSET, S., PILLOT, C., COCHOIS, V., SCHWARTZ, J.-C. & ARRANG, J.-M. (2000). Cloning and cerebral expression of the guinea pig histamine H_3-receptor: evidence for two isoforms. *Neuroreport,* **11,** 755–759.

TOZER, M.J. & KALINDJIAN, S.B. (2000). Histamine H_3 receptor antagonists. *Exp. Opin. Ther. Patents,* **10,** 1045–1055.

WATT, G.F., SYKES, D.A., ROBERTS, S.P., SHANKLEY, N.P. & BLACK, J.W. (1997). Estimation of agonist affinity and efficacy parameters of histamine H_3-receptor ligands in guinea-pig ileum. *Br. J. Pharmacol.,* **122,** 435P.

WERTHWEIN, S., BAUER, U., NAKAZI, M., KATHMANN, M. & SCHLICKER, E. (1999). Further characterization of the ORL_1 receptor-mediated inhibition of noradrenaline release in the mouse brain in vitro. *Br. J. Pharmacol.,* **127,** 300–308.

(Received October 2, 2000
Revised January 15, 2001
Accepted February 1, 2001)