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Characterization of histamine-H_3 receptors controlling non-adrenergic non-cholinergic contractions of the guinea-pig isolated ileum

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1 In the presence of atropine, mepyramine and ranitidine, electric field stimulation of the guinea-pig isolated ileum longitudinal muscle-myenteric plexus preparation resulted in a two component non-adrenergic non-cholinergic contraction. The initial contraction had a duration of approximately 1 s whereas the second contraction lasted approximately 10 s. The second contraction was completely inhibited by tetrodotoxin (0.2×10^{-6} M) with minimal effect on the initial contraction. Phentolamine (3×10^{-6} M), propranolol (3×10^{-6} M) and hexamethonium (10^{-4} M), did not significantly reduce either component of the contractile response.

2 The neurokinin NK_1 receptor antagonists, GR82334 and GR71251, produced concentration-related (EC_{50} = 564 and 173 nM respectively) inhibitions of the second contraction with no effect on the initial contraction. The neurokinin NK_2 receptor antagonists MEN 10207 and Ac-Leu-Asp-Gln-Trp-Phe-Gly-NH_2 (R 396), 1×10^{-9}–10^{-5} M, were without effect on either component of the contractile response.

3 Concentration-related inhibitions of the second contraction, with no effect on the initial contraction, were observed after inclusion of the histamine H_3 receptor agonists (**R**)-α-methylhistamine (pD_2 = 7.6), N^{α}-methylhistamine (pD_2 = 7.7) and N^{α},N^{α}-dimethylhistamine (pD_2 = 6.3). Histamine also inhibited the second contraction (pD_2 = 6.2) in a concentration-related manner but produced a lower maximum inhibitory effect than the other agonists tested.

4 Inclusion of the H_3 receptor antagonists, thioperamide, burimamide, impromidine and phenylbutanoylhistamine, caused parallel concentration-related rightward shifts in the concentration-response curve to (**R**)-α-methylhistamine. In each case, Schild analysis of these data gave slopes not significantly different from unity. Antagonist affinity values for thioperamide (pA_2 = 8.2), burimamide (pA_2 = 7.0) and impromidine (pA_2 = 7.0) were consistent with values obtained in other assays of the H_3 receptor. However, phenylbutanoylhistamine (pA_2 = 5.8) and betahistine ($pK_B \leq 4$) had affinities more than ten fold lower than values obtained in other assays of the H_3 receptor.

5 Exposure of the tissues to N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (10^{-6} M) for 7–30 min followed by extensive washing, had no effect on basal contractions, but produced a rightward shift in the concentration-response curves to (**R**)-α-methylhistamine, N^{α}-methylhistamine, N^{α},N^{α}-dimethylhistamine and histamine. This treatment also resulted in a decrease in the maximum inhibitory response obtainable. Apparent agonist affinity (pK_D) values of 7.01, 7.06, 6.09 and 6.13 were estimated for (**R**)-α-methylhistamine, N^{α}-methylhistamine, N^{α},N^{α}-dimethylhistamine and histamine respectively.

6 In conclusion, pharmacological analysis has revealed that histamine H_3 receptors in the guinea-pig ileum modulate the release of non-adrenergic non-cholinergic neurotransmitters, one of which is probably substance P. In addition we have identified N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline as an irreversible antagonist at H_3 receptors and have used this compound to estimate apparent affinity values of agonists at H_3 receptors in this preparation.

Keywords: Guinea-pig ileum; histamine H_3 receptors; non-adrenergic non-cholinergic contractions

Introduction

The effects of histamine are thought to be mediated through H_1, H_2, and H_3 receptors. The most recently discovered of these, the H_3 receptor, was first described by Arrang *et al.* (1983) as a presynaptic receptor, regulating neuronal release and synthesis of histamine in slices of rat cortex. The most potent and selective H_3 receptor agonist described to date is the chiral compound, (**R**)-α-methylhistamine (RAMH) (Arrang *et al.*, 1987); other H_3 receptor agonists include histamine itself and a number of other methylated derivatives of histamine, notably N^{α}-methylhistamine (NAMH) and N^{α},N^{α}-dimethylhistamine (NADMH) (Arrang *et al.*, 1983). Antagonists at H_3 receptors include the highly selective and potent compound, thioperamide (Arrang *et al.*, 1987), the H_2 receptor antagonist burimamide (Arrang *et al.*, 1983), the H_2 receptor agonist impromidine (Arrang *et al.*, 1983), phenylbutanoylhistamine (Lipp *et al.*, 1988) and betahistine (Arrang *et al.*, 1985).

Since their discovery, it has substantially been shown that presynaptic H_3 receptors can regulate release of neurotransmitters other than histamine in both the central and peripheral nervous systems. Thus, in the brain, activation of H_3 receptors has been shown to inhibit release of noradrenaline (Schlicker *et al.*, 1989), and 5-hydroxytryptamine (Schlicker *et al.*, 1988), whilst in the periphery, H_3 receptor activation inhibits release of acetylcholine (Ichinose *et al.*, 1989) and neurotransmitters of the non-adrenergic non-cholinergic (NANC) type (Ichinose & Barnes, 1989).

Trzeciakowski (1987) and Hew *et al.* (1990) demonstrated that activation of H_3 receptors in the guinea-pig isolated ileum inhibited contractions of the smooth muscle mediated by electrically-stimulated release of acetylcholine. Prior to this, Ambache & Zar (1970) described an inhibitory action of histamine on NANC contractions within this preparation but

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only in the presence of H_1 receptor antagonists. Furthermore, they found that the inhibitory action of histamine could be blocked by burimamide at concentrations much lower than that needed for effective H_2 receptor blockade (Ambache *et al.*, 1973) and close to those now known to be required to block the H_3 receptor. Thus, in addition to inhibiting cholinergically-mediated contractions of the guinea-pig isolated ileum, it would appear that activation of H_3 receptors in this preparation also inhibits contractions mediated by NANC neurotransmitter(s). The main aim of this study was to explore this possibility and, by use of the known H_3 receptor agonists and antagonists described earlier, to characterize the H_3 receptor mediating inhibition of NANC contractions of the guinea-pig isolated ileum.

Additional aims of this study were to examine the effects of neurokinin receptor antagonists on the NANC contractions and also to investigate the effect of the alkylating agent N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) on H_3 receptor agonist-mediated inhibitions of the NANC contractions.

Methods

Tissue preparation

Male Dunkin Hartley guinea-pigs (*Porcellus*) weighing 300–350 g were humanely killed by cervical dislocation. A 10 cm section of ileum containing mesentery was immediately removed 10 cm from the ileo-caecal junction and placed in Krebs-Henseleit solution aerated with 95% O_2: 5% CO_2 (Krebs Henseleit solution composition (mM): NaCl 118, KCl 4.7, $NaHCO_3$ 25, KH_2PO_4 1.18, $MgSO_4 \cdot 7H_2O$ 1.18, $CaCl_2$ 2.5 and glucose 11). Longitudinal muscle strips containing the myenteric plexus were prepared from 3 cm portions of the ileum by the method of Rang (1964). The strips of muscle were transferred to 5 ml organ baths and bathed in Krebs-Henseleit solution containing atropine sulphate 1×10^{-7} M, mepyramine maleate 1×10^{-6} M and ranitidine hydrochloride 1×10^{-5} M, aerated with 95% O_2: 5% CO_2 and maintained at 37 ± 1°C. The tissues were placed under a resting tension of 0.5 g and contractions were recorded isometrically. Once mounted in the organ baths, the muscle strips were subjected to an electric field stimulation delivered via electrodes above and beneath the preparation. The tissues were stimulated every minute by a train of 20 pulses of 100 Hz frequency, 0.5 ms pulse duration and supramaximal intensity of 30 V. Maximal consistent contractions were obtained routinely within 1 h, and during this time the Krebs-Henseleit solution in the organ baths was replaced every 10 min.

Drug applications

Addition of phentolamine, propranolol, tetrodotoxin, hexamethonium, neurokinin receptor antagonists and H_3 receptor agonists The effects of phentolamine (3×10^{-6} M), propranolol (3×10^{-6} M), tetrodotoxin (TTX, 0.2×10^{-6} M), hexamethonium (1×10^{-4} M), the neurokinin NK_1 receptor antagonists GR82334 (1×10^{-10}–3×10^{-6} M) and GR71251 (1×10^{-10}–10^{-5} M), the neurokinin NK_2 receptor antagonists MEN 10207 (1×10^{-8}–10^{-5} M) and Ac-Leu-Asp-Gln-Trp-Phe-Gly-NH_2 (R 396) (1×10^{-9}–10^{-5} M), and the H_3 receptor agonists RAMH, NAMH, NADMH (1×10^{-9}–10^{-5} M) and histamine (1×10^{-9}–10^{-4} M) on the electrically-mediated NANC contractions were investigated only when a regular pattern of reproducible contractile responses was observed. All compounds were added directly to the bath in a 50 μl volume and where a range of drug concentrations was tested, the drug was added in a cumulative fashion, such that the total volume added did not exceed 7% of the bath volume. The effect of each drug concentration (except tetrodotoxin) was examined for 3 min before either washing the tissue or applying the next concentration of drug: the effect of tetrodotoxin was examined for 10 min.

Addition of H_3 receptor antagonists and EEDQ The actions of the H_3 receptor antagonists thioperamide (1×10^{-8}–10^{-5} M), burimamide (1×10^{-7}–10^{-5} M), impromidine (3×10^{-7}–3×10^{-6} M), phenylbutanoylhistamine (1×10^{-5}–10^{-4} M) and betahistine (1×10^{-6}–10^{-4} M) were investigated with RAMH used as the agonist. In each case a concentration-effect curve to RAMH was produced, the tissues washed and contractions allowed to recover. To investigate the action of the known H_3 receptor antagonists, the compounds were allowed to equilibrate with the tissue for 30 min before repeating the RAMH concentration-effect curve in the presence of antagonist. The effect of the H_3 receptor antagonists was investigated at a minimum of three different concentrations.

The effects of EEDQ were investigated with RAMH, NAMH, NADMH and histamine as agonists. In experiments where NAMH, NADMH and histamine were used as agonists the concentration of mepyramine in the Krebs-Henseleit solution was increased to 1×10^{-5} M. For each agonist, a concentration-effect curve to the particular agonist was produced, the tissues washed and contractions allowed to recover. The effect of EEDQ was investigated by exposing the tissues to this compound for various times, followed by extensive washing and repetition of the agonist concentration-effect curve.

Chemicals

Atropine sulphate, histamine dihydrochloride, (S)-(−)-propranolol hydrochloride, tetrodotoxin and hexamethonium bromide were purchased from Sigma Chemical Company Ltd (St. Louis, U.S.A.), phentolamine mesylate from CIBA Laboratories Ltd (Sussex), betahistine dihydrochloride and EEDQ from Aldrich Chemical Company Ltd. (U.K.), and N^{α}-methylhistamine from Calbiochem Corporation (U.S.A.). Gifts of N^{α},N^{α}-dimethylhistamine, burimamide and impromidine trihydrochloride (SmithKline Beecham) and mepyramine maleate (Rhone Poulenc) are gratefully acknowledged. Thioperamide, (R)-α-methylhistamine hydrochloride, ranitidine hydrochloride, phenylbutanoylhistamine, GR71251 ([D-Pro^9[Spiro-γ-Lactam]Leu^{10},Trp^{11}]substance P (1-11)), GR82334 ([D-Pro^9[Sprio-γ-Lactam]Leu^{10},Trp^{11}]physalaemin (1-11)), Ac-Leu-Asp-Gln-Trp-Phe-Gly-NH_2 (R 396) and Asp-Tyr-Trp-Val-Trp-Arg-NH_2 (MEN 10207) were synthesized by the Chemistry Research Department, Glaxo Group Research.

Analysis of data

The effects of all the drugs used in this study were determined by analysing chart recorder tracings of tissue contractile responses. The effect of all drugs was assessed by comparing the average height of three control NANC contractions (taken immediately prior to drug application) with the average height of the three NANC contractions of the tissue in the presence of the drug. The effect of the drug was expressed as a % inhibition of the NANC contractions (where 100% represented complete inhibition).

For H_3 receptor agonists and neurokinin receptor antagonists, EC_{50} values and maxima of concentration-effect curves were estimated with the curve-fitting programme 'Allfit' (De Lean *et al.*, 1977). Apparent agonist affinity (pK_D) values were estimated by the null method of Furchgott using a weighted non-linear curve-fitting programme (McPherson *et al.*, 1983).

H_3 receptor antagonist-induced parallel displacement of RAMH concentration-effect curves was quantified as the ratio

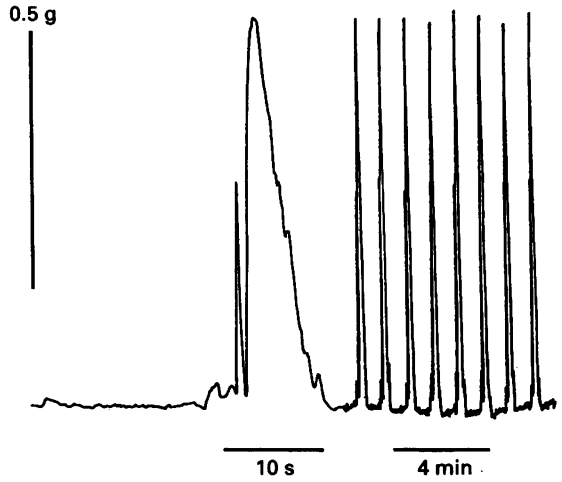

Figure 1 Electrically-mediated non-cholinergic contractions of the guinea-pig isolated ileum longitudinal muscle-myenteric plexus preparation. Once mounted in organ baths, the tissues were subjected to an electric field stimulation delivered via electrodes above and beneath the preparation. Tissues were stimulated every minute by a train of 20 pulses of 100 Hz frequency, 0.5 ms pulse duration and supramaximal intensity of 30 V.

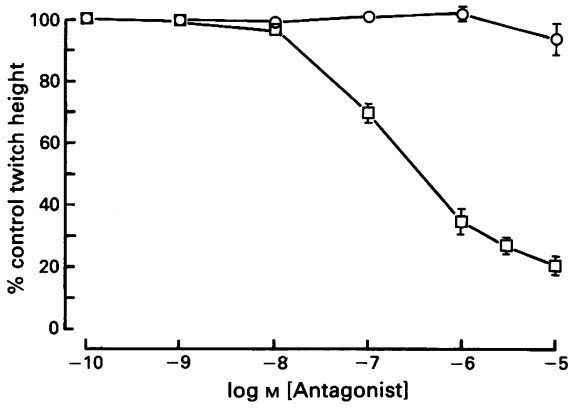

Figure 2 Effect of a neurokinin NK_1 and NK_2 receptor antagonist on NANC contractions of the guinea-pig ileum longitudinal muscle-myenteric plexus preparation. The neurokinin NK_1 receptor antagonist, GR71251 (□), and the neurokinin NK_2 receptor antagonist, MEN 10207 (○) were added to the Krebs-Henseleit solution bathing the tissues in a cumulative fashion such that the total volume added did not exceed 7% of the bath volume. Each point is the mean, with vertical lines indicating the s.e.mean, of determinations in at least four separate preparations, each obtained from a different animal.

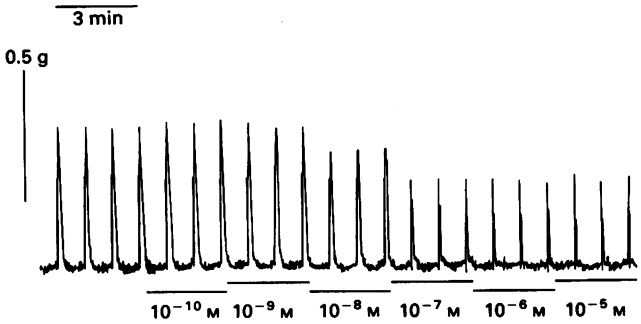

Figure 3 Effect of the H_3 receptor agonist (R)-α-methylhistamine on NANC contractions of the guinea-pig ileum longitudinal muscle-myenteric plexus preparation. Each concentration of (R)-α-methylhistamine was added in a volume of 50 μl directly to the Krebs-Henseleit solution bathing the tissues.

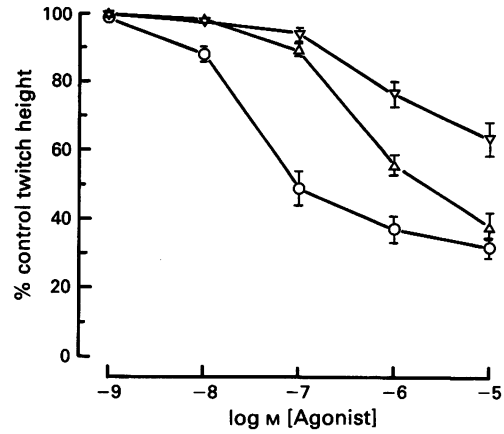

Figure 4 Effect of the H_3 receptor agonists (R)-α-methylhistamine (○), N^{α},N^{α}-dimethylhistamine (△) and histamine (▽) on the NANC contractions of the guinea-pig ileum longitudinal muscle-myenteric plexus preparation. Each point is the mean, with vertical lines indicating the s.e.mean, of determinations in at least five separate preparations each obtained from a different animal.

of equi-active molar concentrations. These values were estimated graphically at the level of the half-maximal response. Antagonist affinity (pA_2 and pK_B) values were estimated with the Schild analysis of these data (Arunlakshana & Schild, 1959). Tissue preparations from at least four different animals (unless indicated otherwise) were used for each drug treatment.

Results

Electrically-mediated contractile responses

In the presence of atropine, the electric field stimulation parameters employed in this study produced contractile responses of the longitudinal muscle preparations which reached maximal tension after 1–1.5 h. Once established, a regular pattern of reproducible contractile responses could be maintained for over 5 h. Individual contractile responses consisted of two discrete contractions: an initial contraction (tension = 0.41 ± 0.04 g; mean ± s.e.mean, $n = 38$) which was rapid in both onset and offset, and a second contraction (tension = 0.58 ± 0.05 g; mean ± s.e.mean, $n = 38$) which lasted for approximately 10 s (Figure 1). The contraction phase of this second contractile component was smooth and progressive whilst the relaxation phase was more complex usually having two or more 'shoulders' (Figure 1).

Effects of phentolamine, propranolol, tetrodotoxin, hexamethonium and neurokinin receptor antagonists

Inclusion of phentolamine (3×10^{-6} M) and propranolol (3×10^{-6} M) in the Krebs-Henseleit solution bathing the tissues resulted, respectively, in a small increase (19 ± 2%) and decrease (8 ± 2%) in the height of the second component of the contractile response with no effect on the initial component. Inclusion of TTX (0.2×10^{-6} M) in the Krebs-Henseleit solution resulted in complete inhibition of the second component of the contractile response within 3 min. The initial component of the contractile response was inhibited maximally by only 20 ± 3%. Inclusion of hexamethonium (1×10^{-4} M) in the Krebs-Henseleit solution had no significant effect on either component of the contractile response.

The effect of inclusion of the neurokinin receptor antagonists in the Krebs-Henseleit solution is shown in Figure 2. Inclusion of either of the neurokinin NK_1 receptor antagonists, GR82334 or GR71251, resulted in a concentration-related inhibition of the second component of the contractile response with no effect on the initial component (Figure 2). Analysis of these data revealed EC_{50} values for GR82334 and GR71251 of 564 ± 75 nM and 173 ± 34 nM respectively, and maximum inhibitory effects of 83 ± 4% and 79 ± 3% respectively. Maximal contractions of the tissues were recovered after two washes with fresh Krebs-Henseleit solution.

Inclusion of the neurokinin NK_2 receptor antagonists, MEN 10207 (1×10^{-8}–10^{-5} M) and R 396 (1×10^{-9}–10^{-5} M) in the Krebs-Henseleit solution had no significant effect on either component of the contractile response (Figure 2).

Effects of H_3 receptor agonists and antagonists

Cumulative additions to the organ bath of increasing concentrations of RAMH resulted in inhibition of the second component of the contractile response with no effect on the initial component (Figures 3 and 4). Similar concentration-related inhibition of the secondary contraction was observed with NAMH, NADMH and histamine, although the apparent maximum inhibitory effect produced by histamine at 1×10^{-5} M was markedly lower than the other H_3 receptor agonists (Figure 4; Table 1): a higher concentration of histamine (1×10^{-4} M) was seen to increase the baseline signal significantly and to cause an increase in the height of the second contraction (results not shown). Agonist potency (pD_2) values and maximal inhibitory effects of the H_3 receptor agonists are shown in Table 1. No change in the maximum inhibitory effect or potency value was apparent on repeated challenge with each agonist (results not shown).

Inclusion of thioperamide (Figure 5a), burimamide (Figure 5b) impromidine and phenylbutanoyl-histamine in the organ baths resulted in concentration-related parallel rightward shifts of the RAMH concentration-response curve, with no effect on the maximal inhibitory effect obtainable. Schild plots constructed with these data showed, in each case, a slope not significantly different from unity. The calculated affinity (pA_2 and pK_B) values for these compounds are shown in Table 2. Inclusion of betahistine in the organ bath had no effect on the RAMH concentration-response curve at any concentration tested (1×10^{-6}–10^{-4} M). A Schild plot could not be constructed from these data and so an affinity (pK_B) value ≤ 4 was estimated for this compound ($n = 12$).

Exposure of the tissues to EEDQ (1×10^{-6} M) for various times (7–30 min), followed by extensive washing, had no effect on either component of the contractile response. However,

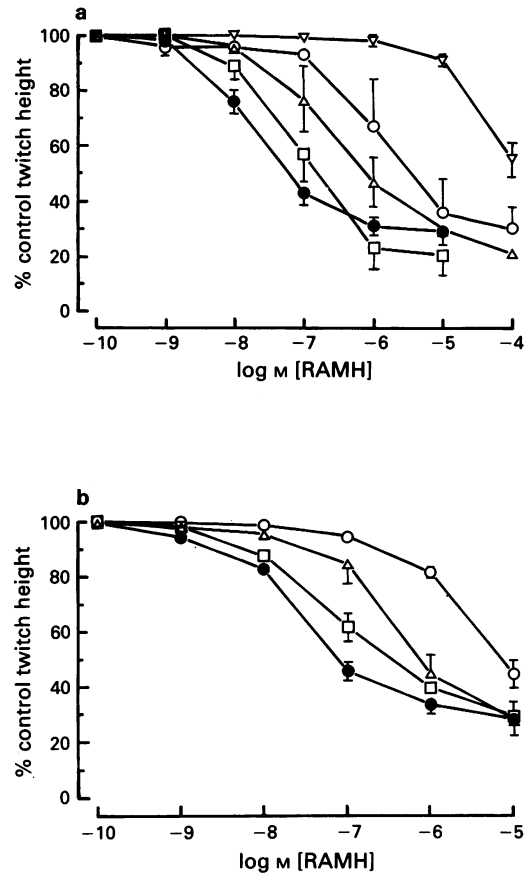

Figure 5 Effect of (a) thioperamide and (b) burimamide on (R)-α-methylhistamine (RAMH)-mediated inhibition of NANC contractions of the guinea-pig ileum longitudinal muscle-myenteric plexus preparation. The 'control' concentration-response curve (●) in each graph was constructed from cumulative additions of RAMH; each preparation was exposed to only one concentration of antagonist. In all graphs each point is the mean, with vertical lines indicating the s.e.mean, of single determinations in at least four separate preparations, each obtained from a different animal. Antagonist concentrations are as follows: thioperamide (□ 10^{-8} M, △ 10^{-7} M, ○ 10^{-6} M, ▽ 10^{-5} M), burimamide (□ 10^{-6} M, △ 10^{-5} M, ○ 3×10^{-5} M).

Table 1 Mean pD_2 values and maximal inhibitions of agonists to inhibit electrically-evoked NANC contractions of the guinea-pig isolated ileum longitudinal muscle-myenteric plexus preparation

Compound	*pD_2*	*Maximum inhibition of NANC contraction obtainable* (%)
NAMH	7.70 ± 0.05	63 ± 1
RAMH	7.64 ± 0.06	72 ± 1
NADMH	6.31 ± 0.05	65 ± 2
Histamine	6.16 ± 0.09	40 ± 3

Each value is the mean (± s.e.mean) of determinations in at least five separate preparations each obtained from a different animal.
Abbreviations: NAMH: N^{α}-methylhistamine; RAMH: (R)-α-methylhistamine; NADMH: N^{α},N^{α}-dimethylhistamine.

Table 2 Apparent affinity values of compounds used to inhibit (R)-α-methylhistamine-mediated inhibition of NANC contractions of the guinea-pig isolated ileum longitudinal muscle-myenteric plexus preparation

Compound	*pA_2*	*Schild slope*	*pK_B*	n
Thioperamide	8.22 ± 0.22	0.95 ± 0.10	8.11 ± 0.11	17
Burimamide	6.98 ± 0.16	0.93 ± 0.08	6.85 ± 0.04	14
Impromidine	6.98 ± 0.38	0.63 ± 0.22	6.60 ± 0.09	12
PBH	5.80 ± 0.21	0.88 ± 0.13	5.64 ± 0.06	12

Each value is the mean (± s.e.mean) of determinations in n separate preparations each obtained from a different animal. At least three different concentrations of each compound were tested. pA_2 values were calculated by use of the equation of Arunlakshana & Schild (1959) and pK_B values were calculated by constraining the slope of this analysis to unity. Abbreviations: PBH; phenylbutanoylhistamine

this treatment had profound actions on the concentration-effect curves to all the agonists tested (Figure 6) (Table 3). For each agonist, increasing the exposure time of the tissues to EEDQ resulted in an increase in the EC_{50} of that agonist and a decrease in the maximum inhibitory effect obtainable. However, exposure of the tissues to EEDQ for 7 min produced an increase in the EC_{50} value of RAMH with no change in the maximum inhibitory effect obtainable. By use of a weighted non-linear curve fitting programme (McPherson *et al.*, 1983) agonist affinity (pK_B) values of 7.01, 7.06, 6.09 and 6.13 were estimated for RAMH, NAMH, NADMH and histamine respectively.

Discussion

The contractile effect of histamine on the guinea-pig isolated ileum preparation has been well characterized (Arunlakshana & Schild, 1959; Ash & Schild, 1966; Leurs *et al.*, 1991) and as such has provided a valuable tool for assaying H_1 receptor activity. However, histamine receptors which mediate inhibition of both cholinergic (Fjalland, 1979) and NANC (Ambache *et al.*, 1973) contractions within this preparation have also been suggested: on the basis of pharmacological data it was proposed that these effects were mediated by a

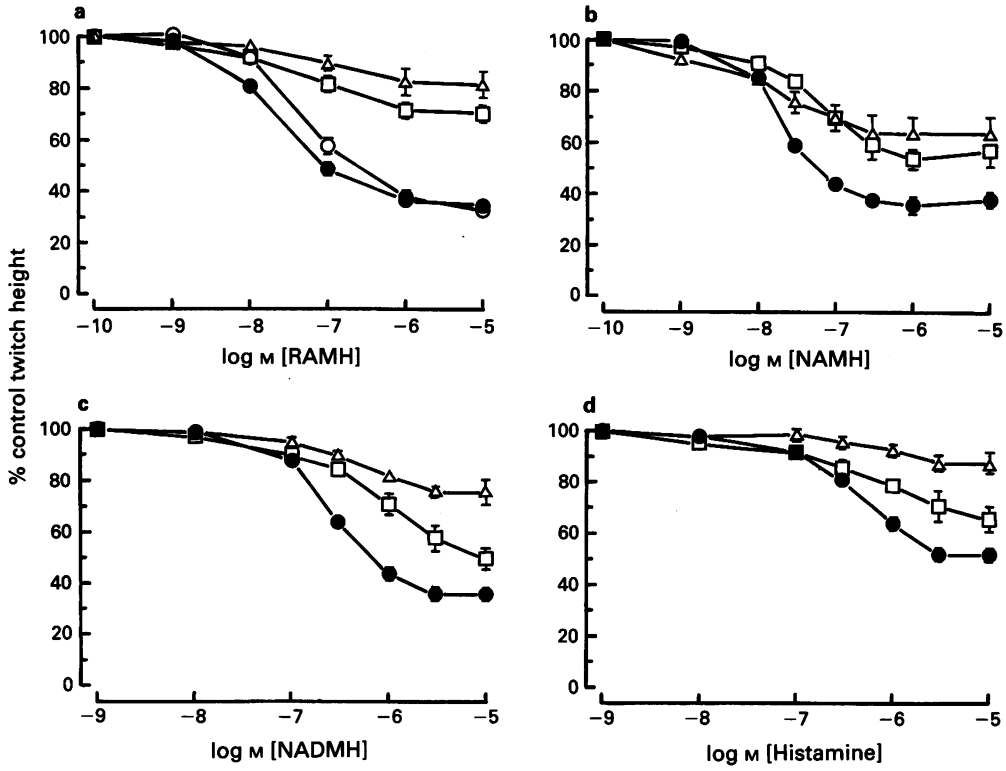

Figure 6 Effect of exposure of tissues to EEDQ (10^{-6} M) on (a) (R)-α-methylhistamine (RAMH) (b) N^{α}-methylhistamine (NAMH), (c) N^{α},N^{α}-dimethylhistamine (NADMH) and histamine-mediated inhibitions (d) of NANC contractions of the guinea-pig ileum longitudinal muscle-myenteric plexus preparation. Each point is the mean, with vertical lines indicating the s.e.mean, of determinations in at least four separate preparations, each obtained from a different animal. EEDQ exposure times are as follows: ● 0 min (control), ○ 7 min, □ 15 min and △ 30 min.

Table 3 Effect of increasing the time of exposure of the tissues to N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ, 1×10^{-6} M) on maximum inhibitory effect and potency of H_3 receptor agonists

Agonist	*Exposure time* (min)	*Maximum inhibition of NANC contraction obtainable* (%)	EC_{50} (nM)	pD_2	K_D (nM)	pK_D (average)	n
RAMH	Control	61.8 ± 1.1	28 ± 3	7.55 ± 0.004	—		18
	7	66.5 ± 0.9	59 ± 4	7.23 ± 0.03	—		6
	15	26.4 ± 1.3	61 ± 15	7.21 ± 0.09	90	7.01	6
	30	16.9 ± 1.9	87 ± 47	7.06 ± 0.19	110		6
NAMH	Control	66.4 ± 1.9	23 ± 3	7.64 ± 0.05	—		10
	15	46.1 ± 1.8	49 ± 9	7.31 ± 0.07	130	7.06	6
	30	37.0 ± 2.1	42 ± 11	7.38 ± 0.10	60		4
NADMH	Control	69.0 ± 1.5	288 ± 26	6.54 ± 0.04	—		13
	15	30.3 ± 1.8	911 ± 205	6.04 ± 0.09	1470	6.09	6
	30	27.0 ± 1.7	400 ± 105	6.40 ± 0.10	460		7
Hist	Control	52.1 ± 2.0	518 ± 75	6.29 ± 0.06	—		12
	15	33.4 ± 2.4	504 ± 146	6.30 ± 0.11	600	6.13	6
	30	13.6 ± 1.8	842 ± 413	6.07 ± 0.17	940		6

Each value is the mean (± s.e.mean) of determinations in *n* separate preparations each obtained in a different animal. Data analysis was carried out by use of a weighted non-linear curve-fitting programme (McPherson *et al.*, 1983). Abbrevations: RAMH; (R)-α-methylhistamine: NAMH; N^{α}-methylhistamine: NADMH; N^{α},N^{α}-dimethylhistamine: Hist; histamine.

subtype of the H_2 receptor. However, doubt was cast on this proposal by the subsequent discovery of the H_3 receptor, and the realization that some H_1 and H_2 receptor ligands are in fact potent antagonists at these receptors. Indeed in 1987, Trzeciakowski provided strong evidence to suggest that the inhibitory action of histamine on cholinergic contractions of the guinea-pig ileum was mediated via H_3 receptors. In this study we have examined NANC contractions within this preparation and have provided evidence to suggest that inhibition of these contractions can also be mediated by activation of H_3 receptors. Indeed, during the preparation of this manuscript, a paper supporting this suggestion was published (Menkveld & Timmerman, 1990).

The atropine-resistant response evoked by electric field stimulation of the guinea-pig isolated ileum preparation in this study comprised an initial fast contraction and a second slow contraction. Neither component of this contractile response was affected to any major degree by the presence of phentolamine and propranolol at concentrations which would have almost completely blocked α- and β-adrenoceptors. These observations suggest that the contractile responses observed could be mediated by direct electrical stimulation of the smooth muscle and/or a non-adrenergic non-cholinergic (NANC) neurotransmitter(s) released by electric field stimulation. Whilst the second component of the contractile response could be completely abolished by TTX, the initial fast component was only inhibited maximally by approximately 20%. These observations suggest that the second contraction is mediated by neuronal release of a NANC neurotransmitter(s) whereas the initial contraction is due, in most part, to direct smooth muscle stimulation. Such a proposal is in good agreement with the recent findings of Menkveld & Timmerman (1990). Furthermore, the lack of effect of hexamethonium on the NANC contraction suggests that release of the NANC neurotransmitter(s) does not occur from preganglionic neurones.

No conclusive evidence has yet been offered as to the nature of the neurotransmitter(s) and receptor(s) involved in the NANC contraction evoked by electric field stimulation of the guinea-pig isolated ileum. However, it has been established that the neurotransmitter is not histamine, 5-hydroxytryptamine or a prostaglandin (Ambache & Freeman, 1968). In this study we have demonstrated that GR71251 and GR82334, two selective neurokinin NK_1 receptor antagonists (Hagan *et al.*, 1989; 1990) inhibited the NANC contractions in a concentration-related manner. In contrast, no inhibition of the NANC contractions was observed using the selective neurokinin NK_2 receptor antagonists MEN 10207 (Maggi *et al.*, 1990) and R 396 (Maggi *et al.*, 1990). The possible function of neurokinin NK_3 receptors in the NANC contraction could not be investigated since no selective neurokinin NK_3 receptor antagonist has yet been reported. Nevertheless, the results suggest that neurokinin NK_1, but not neurokinin NK_2 receptors have an important role in the mediation of the NANC contraction of the guinea-pig isolated ileum. This observation provides some clues albeit indirect as to the nature of the neurotransmitter mediating the NANC contraction: of the neurokinins, substance P is known to be the most potent at neurokinin NK_1 receptors (Ireland *et al.*, 1988) and so it would seem likely that this neurotransmitter is one of the hitherto unidentified NANC neurotransmitters in this tissue preparation.

We have demonstrated that all of the H_3 receptor agonists used in this study could inhibit, in a concentration-dependent fashion, the secondary component of the contractile response (the NANC contraction) with no effect on the initial fast component. The lower maximum inhibitory effect observed for histamine is probably due to H_1 or H_2 receptor-mediated contraction of the longitudinal muscle, caused by histamine overcoming the blocking actions of mepyramine and ranitidine. This idea is supported by the observation that a higher concentration of histamine (1×10^{-4} M) significantly increased the baseline signal and actually caused a decrease in the inhibitory effect seen (results not presented). In the light of the earlier findings of this study, the mechanism of action of the H_3 receptor agonists is presumably to activate H_3 receptors located on nerve terminals containing substance P thereby inhibiting its release. The potency values for the agonists tested in this preparation are consistent with the values obtained for these compounds to inhibit [^{3}H]-histamine release from rat cortical slices (Arrang *et al.*, 1987) and the rank order of potency is identical to that reported by Menkveld & Timmerman (1990).

Conclusive evidence that inhibition of the NANC contractions was mediated via H_3 receptor activation comes from the observations made with known H_3 receptor antagonists. The inhibitory response mediated by RAMH was blocked by thioperamide, burimamide, impromidine and phenylbutanoylhistamine but not, to any significant extent, by betahistine. The antagonistic actions of thioperamide, burimamide, impromidine and phenylbutanoylhistamine appeared to be competitive because Schild analysis of the effects of these compounds revealed slopes not significantly different from unity (although the apparent slope for impromidine was low). The affinity values obtained for thioperamide, burimamide and impromidine in other assays of the H_3 receptor (Arrang *et al.*, 1987; Kilpatrick & Michel, 1991) are in good agreement with the affinity values obtained for these compounds in this preparation. However, the affinity value of phenylbutanoylhistamine for H_3 receptors in this preparation ($pA_2 = 5.8$) was more than ten fold lower than the value obtained for this compound in a receptor binding ($pK_i = 6.8$; Kilpatrick & Michel, 1991) and functional ($pK_i = 7.1$; see Timmerman, 1990) assay of the H_3 receptor. Such a discrepancy was even more marked for betahistine.

The reasons for the discrepancies in antagonist affinity are currently under investigation in our laboratory. However, a simple species-difference in H_3 receptors can be discounted in this case because both phenylbutanoylhistamine and betahistine have been shown to be equipotent at inhibiting [^{3}H]-RAMH binding to H_3 receptors in homogenates of guinea-pig and rat cortex (Kilpatrick & Michel, 1991). Possible explanations might therefore include a reduction in the effective concentration of the compounds at H_3 receptors, caused by poor accessibility and/or breakdown of the compounds within the organ bath, or, sub-types of the H_3 receptor.

EEDQ has previously been reported to act as an irreversible antagonist at various mono-amine receptors in peripheral tissues and brain. EEDQ irreversibly blocks binding to α-adrenoceptors in peripheral tissues (Belleau *et al.*, 1968) and blocks binding to D_1 and D_2 dopamine receptors (Hamblin & Creese, 1983) and 5-HT_2 receptors (Battaglia *et al.*, 1986) in brain after peripheral administration. EEDQ has also been reported to act as an irreversible antagonist at muscarinic receptors in peripheral tissues *in vitro* (Chang *et al.*, 1970). The mechanism of action of EEDQ is thought to involve the activation of peptide carboxyl groups so creating a highly reactive mixed carbonic anhydride (Belleau *et al.*, 1969). In a receptor protein this reactive group may then interact with suitable nucleophilic groups, such as adjacent α-amino groups, thus forming an irreversible covalent crosslinking of peptide chains within the receptor (Belleau *et al.*, 1969).

In this study we have demonstrated that exposure of the tissues to EEDQ resulted in a rightward shift in the agonist concentration-effect curve, with a reduction in the maximal inhibitory effect obtainable. This action of EEDQ increased as the exposure time of the tissues to EEDQ was increased. For each of the agonists tested, the apparent dissociation constants (K_D) estimated at the different exposure times were found to be consistent. The effects of EEDQ (i.e. increased EC_{50}, reduced maximal effect obtainable and an identical dissociation constant) are characteristic of a compound that acts as an irreversible antagonist. As such, these results strongly indicate that EEDQ acts as an irreversible antagonist at H_3 receptors within this preparation. It is noteworthy that exposure of the tissues to EEDQ for up to 45 min had no effect on

basal NANC contractions. This observation would perhaps indicate that EEDQ does not act as an irreversible antagonist at neurokinin NK_1 receptors, although it is possible that if the concentration of EEDQ and/or exposure time had been increased, a reduction in the height of the NANC contractions would then have been apparent.

The relative affinity values of the H_3 receptor agonists is very similar to the relative potency of these compounds in this preparation. As the potency of an agonist is the product of its affinity and efficacy, this observation reveals that the H_3 receptor agonists used in this study have approximately the same efficacy at H_3 receptors in this preparation. However, one should be cautious in interpreting the results using EEDQ as it is clear that inaccuracies can occur when estimating agonist affinity values (see Leff, 1988; Leff *et al.*, 1990).

The apparent affinity (pK_D) values calculated for RAMH, NAMH, NADMH and histamine at H_3 receptors in this preparation, are significantly lower than the affinity (pK_i) values obtained for these compounds to inhibit [^{3}H]-RAMH binding to homogenates of rat cortex (Arrang *et al.*, 1987; West *et al.*, 1990; Kilpatrick & Michel, 1991). However, such a difference is not unexpected when making a comparison of agonist affinities between a functional and a receptor binding assay because many receptors are thought to exist in two agonist affinity states: a functionally important low affinity state and a high affinity state. A radioactive agonist ligand is thought to label selectively the high affinity state of the receptor and so higher agonist affinity values would be expected in a receptor binding assay than a functional assay.

In summary, this study demonstrates that activation of H_3 receptors within the guinea-pig ileum, can inhibit NANC contractions elicited by electric field stimulation: the NANC contraction is probably mediated by substance P acting at neurokinin NK_1 receptors. In addition, we have identified an irreversible antagonist at H_3 receptors within this preparation and as such this compound should become a useful tool for both *in vivo* and *in vitro* study of H_3 receptors. Finally, we feel that this preparation provides a rapid and simple functional assay for investigating the H_3 receptor, and for determining agonist and antagonist activity at this receptor.

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References

AMBACHE, N. & ZAR, M.A. (1970). An inhibitory action of histamine on the guinea-pig ileum. *Br. J. Pharmacol.*, **38**, 229–240.

AMBACHE, N. & FREEMAN, A.M. (1968). Atropine-resistant longitudinal muscle spasms due to excitation of non-cholinergic neurones in Auerbach's plexus. *J. Physiol.*, **199**, 705–727.

AMBACHE, N., KILLICK, S.W. & ZAR, M.A. (1973). Antagonism by burimamide of inhibitions induced by histamine in plexus-containing longitudinal muscle preparations from guinea-pig ileum. *Br. J. Pharmacol.*, **48**, 362–363P.

ARRANG, J.M., GARBARG, M., LANCELOT, J.C., LECOMTE, J.M., POLLARD, H., ROBBA, M., SCHUNACK, W. & SCHWARTZ, J.C. (1987). Highly potent and selective ligands for histamine H_3 receptors. *Nature*, **327**, 117–123.

ARRANG, J.M., GARBARG, M., QUACH, T.T., DAM TRUNG TUONG, T., YERAMIAN, E. & SCHWARTZ, J.C. (1985). Actions of betahistine at histamine receptors in the brain. *Eur. J. Pharmacol.*, **111**, 73–84.

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.

ARUNLAKSHANA, O. & SCHILD, H.O. (1959). Some quantitative uses of drug antagonists. *Br. J. Pharmacol. Chemother.*, **14**, 153–161.

ASH, A.S.F. & SCHILD, H.O. (1966). Receptors mediating some actions of histamine. *Br. J. Pharmacol.*, **27**, 427–439.

BATTAGLIA, G., NORMAN, A.B., NEWTON, P.L. & CREESE, I. (1986). *In vitro* and *in vivo* irreversible blockade of cortical S_2 serotonin receptors by N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline: a technique for investigating S_2 serotonin receptor recovery. *J. Neurochem.*, **46**, 589–593.

BELLEAU, B., DITULLIO, V. & GODIN, D. (1969). The mechanism of irreversible blockade by N-carbethoxydihydroquinolines—model studies with typical serine hydrolases. *Biochem. Pharmacol.*, **18**, 1039–1044.

BELLEAU, B., MASTEL, R., LACASSE, G., MENARD, M., WEINBERG, N.L. & PERRON, Y.G. (1968). N-carboxylic acid esters of 1,2 and 1,4-dihydroquinolines. A new class of irreversible inactivators of the catecholamine alpha receptors and potent central nervous system depressants. *J. Am. Chem. Soc.*, **90**, 823–824.

CHANG, K.J., MORAN, J.F. & TRIGGLE, D.J. (1970). Mechanism of cholinergic antagonism by N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ). *Pharmacol. Res. Commun.*, **2**, 63–66.

DE LEAN, A., MUNSON, P.J. & RODBARD, D. (1977). Simultaneous analysis of families of sigmoidal curves: application to bioassay, radioligand assay and physiological dose-response curves. *Am. J. Physiol.*, **235**, E97–E102.

FJALLAND, B. (1979). Evidence for the existence of another type of histamine H_2-receptor in guinea-pig ileum. *J. Pharm. Pharmacol.*, **31**, 50–51.

HAGAN, R.M., IRELAND, S.J., JORDAN, C.C., BAILEY, F., STEPHENS-SMITH, M.L., DEAL, M. & WARD, P. (1989). Novel, potent and selective agonists at NK-1 and NK-2 receptors. *Br. J. Pharmacol.*, **98**, 717P.

HAGAN, R.M., IRELAND, S.J., JORDAN, C.C., BERESFORD, I.J.M., STEPHENS-SMITH, M.L., EWAN, G. & WARD, P. (1990). GR71251, A novel, potent and highly selective antagonist at neurokinin NK-1 receptors. *Br. J. Pharmacol.*, **99**, 62P.

HAMBLIN, M. & CREESE, I. (1983). Behavioural and radioligand binding evidence for irreversible dopamine receptor blockade by N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline. *Life Sci.*, **32**, 2247–2255.

HEW, R.W.S., HODGKINSON, C.R. & HILL, S.J. (1990). Characterization of histamine H_3 receptors in guinea-pig ileum with H_3 selective ligands. *Br. J. Pharmacol.*, **101**, 621–624.

ICHINOSE, M. & BARNES, P.J. (1989). Histamine H_3-receptors modulate non-adrenergic non-cholinergic neural bronchoconstriction in guinea-pig *in vivo*. *Eur. J. Pharmacol.*, **174**, 49–55.

ICHINOSE, M., STRETTON, C.D., SCHWARTZ, J.C. & BARNES, P.J. (1989). Histamine H_3 receptors inhibit cholinergic neurotransmission in guinea-pig airways. *Br. J. Pharmacol.*, **97**, 12–15.

IRELAND, S.J., JORDAN, C.C., STEPHENS-SMITH, M.L. & WARD, P. (1988). Receptors mediating the contractile response to neurokinin agonists in the guinea-pig trachea. *Regul. Pept.*, **22**, 93.

KILPATRICK, G.J. & MICHEL, A.D. (1991). Characterization of the specific binding of the H_3 receptor agonist [^{3}H](R)-α-methyl-histamine. *Agents Actions*, Suppl., 33, 69–75.

LEFF, P. (1988). Analysis of agonist action using the operational model. *Trends Pharmacol. Sci.*, **9**, 395–398.

LEFF, P., DOUGALL, I.G., HARPER, D.H. & DAINTY, I.A. (1990). Errors in agonist affinity estimation: do they and should they occur in isolated tissue experiments. *Trends Pharmacol. Sci.*, **11**, 64–67.

LEURS, R., BROZIUS, M.M., SMIT, M.J., BAST, A. & TIMMERMAN, H. (1991). Effects of histamine H_1-, H_2- and H_3-receptor selective drugs on the mechanical activity of the guinea-pig small and large intestine. *Br. J. Pharmacol.*, **102**, 179–185.

LIPP, R., SCHUNACK, W., ARRANG, J.M., GARBARG, M. & SCHWARTZ, J.C. (1988). Synthesis and H_3-antagonistic activity of N^{α}-susbtituted histamine derivatives. In *Abstracts of the Xth International Symposium on Medicinal Chemistry*. Abstract P-119, Hungarian Chemical Society, MTESZ, Budapest.

MAGGI, C.A., PATACCHINI, R., GUILIANI, S., ROVERO, P., DION, S., REGOLI, D., GIACHETTI, A. & MELI, A. (1990). Competitive antagonists discriminate between NK-2 tachykinin receptor types. *Br. J. Pharmacol.*, **100**, 588–592.

McPHERSON, G.A., MOLENAAR, P., RAPER, C. & MALTA, E. (1983). Analysis of dose-response curves and calculation of agonist dissociation constants using a weighted non-linear curve-fitting program. *J. Pharmacol. Methods*, **10**, 231–241.

MENKVELD, G.J. & TIMMERMAN, H. (1990). Inhibition of electrically evoked contractions of guinea-pig ileum preparations mediated by the histamine H_3 receptor. *Eur. J. Pharmacol.*, **186**, 343–347.

RANG, H.P. (1964). Stimulant actions of volatile anaesthetics on smooth muscle. *Br. J. Pharmacol. Chemother.*, **22**, 356–365.

SCHLICKER, E., BETZ, R. & GOTHERT, M. (1988). Histamine H_3 receptor-mediated inhibition of serotonin release in the rat brain cortex. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **337**, 588–590.

SCHLICKER, E., FINK, K., HINTERTHANER, M. & GOTHERT, M. (1989). Inhibition of noradrenaline release in the rat brain cortex

via presynaptic H_3 receptors. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **340**, 633–638.

TIMMERMAN, H. (1990). Histamine H_3 ligands: just pharmacological tools or potential therapeutic agents? *J. Med. Chem.*, **33**, 4–11.

TRZECIAKOWSKI, J.P. (1987). Inhibition of guinea-pig ileum contractions mediated by a class of histamine receptors resembling the H_3-subtype. *J. Pharmacol. Exp. Ther.*, **243**, 874–880.

WEST, R.E., ZWEIG, A., GRANZOW, R.T., SIEGEL, M.I. & EGAN, R.W. (1990). Bioexponential kinetics of (R)-α[^{3}H]methylhistamine binding to the rat brain H_3 histamine receptor. *J. Neurochem.*, **55**, 1612–1616.

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