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Characterization of histamine H_3 receptors regulating acetylcholine release in rat entorhinal cortex

[1]J.M. Arrang, G. Drutel & J.-C. Schwartz

Unité de Neurobiologie et Pharmacologie (U. 109) de l'INSERM, Centre Paul Broca, 2ter rue d'Alésia, 75014 Paris, France

1 The pharmacological properties and location of H_3 receptors modulating acetylcholine release have been investigated in non-superfused slices and synaptosomes of rat entorhinal cortex preloaded with [^{3}H]-choline.

2 (R)α-methylhistamine, an H_3-receptor agonist, potently inhibited the K^+-evoked tritium release from slices, an effect antagonized by thioperamide, an H_3-receptor antagonist, with nanomolar potency.

3 The K^+-evoked tritium release from synaptosomes remained unaltered in the presence of the potent and selective H_3-receptor agonists, imetit and (R)α-methylhistamine, suggesting that H_3 receptors modulating acetylcholine release are not presynaptically located on cholinergic nerve terminals.

4 Phenylbutanoylhistamine and phenylpropylhistamine, two H_3-receptor antagonists of moderate potency, failed to antagonize the inhibitory effects of (R)α-methylhistamine observed in slices. Unexpectedly, both compounds when used alone, inhibited tritium release from slices and synaptosomes with micromolar potency and to the same extent (by approximately 50% when added at a final concentration of 200 μM). This inhibitory effect did not involve H_1, H_2 or H_3 receptors and was not mediated by an unknown histamine receptor site, since histamine used at a high concentration neither reproduced nor antagonized the effect of phenylbutanoylhistamine. It remained unaltered in the presence of scopolamine and was neither mimicked nor antagonized by vasoactive intestinal peptide, previously shown to be colocalized with acetylcholine in some neurones.

5 It is concluded that acetylcholine release in rat entorhinal cortex is modulated by H_3 receptors presumably not located on cholinergic axon terminals. It remains to be established whether these H_3 receptors belong to a receptor subtype different from those previously described since the potency of phenylbutanoylhistamine and phenylpropylhistamine as H_3-receptor antagonists was probably greatly underestimated by additional properties of both drugs.

Keywords: Acetylcholine release; entorhinal cortex; slices; synaptosomes; histamine H_3 receptors

Introduction

The histamine H_3 receptor was initially detected as an autoreceptor modulating histamine synthesis and release in the brain (Arrang *et al.*, 1983; 1985; 1987a,b). However, autoradiography of H_3 receptors in rat and monkey brain together with lesion experiments (Martinez Mir *et al.*, 1990; Cumming *et al.*, 1991; Pollard *et al.*, 1993) indicate that the majority of central H_3 receptors are not autoreceptors. In agreement with this view, functional studies performed in brain have indicated the occurrence of presynaptic inhibitory H_3 receptors on axon terminals not only of histaminergic but also noradrenergic (Schlicker *et al.*, 1989), 5-hydroxytryptaminergic (Fink *et al.*, 1990), dopaminergic (Schlicker *et al.*, 1993) and peptidergic neurones (Matsubara *et al.*, 1992). Also, it has been recently demonstrated that activation of H_3 receptors inhibits the release of tritium, presumably [^{3}H]-acetylcholine, from slices of rat entorhinal cortex preloaded with [^{3}H]-choline (Clapham & Kilpatrick, 1992) and the pharmacological profile of this response led the authors to postulate the existence of H_3-receptor subtypes. The existence of multiple H_3-receptor subtypes was also suggested based upon the comparison of functional and radioligand binding studies (Arrang *et al.*, 1990; West *et al.*, 1990a,b; Hey *et al.*, 1992; Schlicker *et al.*, 1992).

We have used non-superfused slices and synaptosomes of entorhinal cortex to reinvestigate this possibility as well as to assess the presynaptic localization of H_3 receptors on acetylcholine-releasing terminals themselves.

[1] Author for correspondence.

Methods

Release from slices

Slices (0.3 mm thick) from the entorhinal cortex of male Wistar rats (180-200 g, Iffa Credo, France) were prepared with a McIlwain tissue chopper and suspended in modified Krebs-Ringer bicarbonate medium (mM): NaCl 120, KCl 0.8, $CaCl_2$ 1.3, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 27.5 and glucose 10, pH 7.4 gassed wtih O_2/CO_2 (95:5). The slices were preincubated for 45 min at 37°C in the presence of 0.1 μM [^{3}H]-choline for [^{3}H]-acetylcholine synthesis. The slices were then washed with fresh Krebs solution (4 × 20 ml) in order to remove excess radioactivity, and transferred to a small basket (Arrang *et al.*, 1983). The basket was subsequently immersed six times (for 10 min periods each) in fresh Krebs solution. After the last washing period, 250 μl aliquots of the slice suspension (1-3 mg of protein) were distributed into tubes, preincubated in each experiment for 5 min in the presence of various drugs tested as histamine agonists or antagonists and subsequently incubated for 2 min with 2, 20 or 30 mM K^+ (final concentration). Incubations were stopped by short centrifugation. The pellets were homogenized in 500 μl 0.01 M HCl and radioactivity present in tissue and medium was measured by liquid scintillation spectrometry.

Release from synaptosomes

A crude synaptosomal fraction was prepared after homogenization of the entorhinal cortex in 0.32 M sucrose at 4°C according to the method of Whittaker (1966). Synaptosomes were suspended in Krebs solution and incubated for 45 min with [^{3}H]-choline as described above for slices. After six

extensive washings by successive centrifugations and suspensions in fresh medium (2 mM K^+), synaptosomes (0.3–1 mg of protein) were distributed into tubes, incubated in each experiment for 5 min in the presence, when required, of the various drugs tested as agonists or antagonists and subsequently incubated for 2 min with 2, 10 or 20 mM K^+ (final concentration). Incubations were ended and radioactivity determined as for slice experiments. Protein contents were determined according to the method of Lowry *et al.* (1951) with bovine serum albumin used as the standard.

Analysis of data

Radioactivity (in d.p.m.) was estimated by liquid scintillation spectrometry at 45% counting efficiency.

Values for tritium release elicited by tissue (slices or synaptosomes) depolarization with high K^+ solutions were calculated in each experiment as the difference between mean tritium levels in high K^+ and 2 mM K^+ media of the same experiment, respectively, and are given as percentages of the total tritium present in tissue plus medium.

For determination of the means and standard errors of EC_{50} and IC_{50} values, the total curves were analysed with an iterative computer least-squares method derived from Parker & Waud (1971) (except Figure 3). The apparent dissociation constant (K_i value) of thioperamide was calculated from its IC_{50} value, assuming a competitive antagonism and neglecting the possible effect of endogenous histamine, according to the equation of Cheng & Prusoff (1973): $K_i = IC_{50}/(1 + S/EC_{50})$, where S represents the concentration of (R)α-methylhistamine (1 μM) and EC_{50} the concentration of (R)α-methylhistamine eliciting a half-maximal inhibitory effect on K^+-evoked release of tritium.

Statistical evaluation of the results was by Student's *t* test. Any significant difference obtained for tritium release was determined from pairs of values (representing the mean ± s.e.mean) obtained from experiments carried out in parallel and calculated as percentages before or after subtraction of the mean basal efflux and was checked by statistical analysis (*t* test) of the same parameters expressed in d.p.m. mg^{-1} protein of tritium measured in the media.

Chemicals and drugs

[^{3}H]-choline chloride (75 Ci $mmol^{-1}$) was purchased from the Radiochemical Centre (Amersham, UK). Drugs and their sources were as follows: mepyramine hydrochloride (Specia, Paris, France), ranitidine hydrochloride (Glaxo, Greenford, UK), burimamide and impromidine trihydrochloride (Smith Kline Beecham, London, UK), (R)α-methylhistamine dihydrogenmaleate and thioperamide (Bioprojet, Paris, France), histamine dihydrochloride, oxotremorine sesquifumarate and scopolamine dihydrochloride (Sigma, St Louis, MO, U.S.A.), vasoactive intestinal polypeptide (VIP) (porcine) and thyrotropin releasing hormone (TRH) (Bachem, Bubendorf, Switzerland). Imetit dihydrobromide was synthesized by Prof. C.R. Ganellin (University College London, England) and phenylbutanoylhistamine and phenylpropylhistamine were synthesized by Prof. W. Schunack (Free University, Berlin, Germany).

Results

Release from slices

The spontaneous efflux of tritium from slices of rat entorhinal cortex into the medium in the presence of 2 mM K^+, 1 μM mepyramine and 10 μM ranitidine represented 2031 ± 338 d.p.m. mg^{-1} protein, i.e. 8.8 ± 0.5% of the total (tissue plus medium) tritium, per 2 min. In the presence of 20 mM K^+, 1 μM mepyramine and 10 μM ranitidine, the release of tritium in the medium represented 14.0 ± 0.7% of the total per 2 min, giving an evoked release of 5.2 ± 0.4% ($n = 16$ separate experiments) over spontaneous efflux. The K^+-induced release was inhibited by (R)α-methylhistamine in a concentration-dependent and saturable manner (Figure 1). The maximal inhibition of release was 39 ± 4% and the EC_{50} was 25 ± 10 nM. As previously reported (Clapham & Kilpatrick, 1992), in the absence of mepyramine and ranitidine, the EC_{50} was increased by about one order of magnitude with a similar maximal inhibition (not shown). Therefore, all the experiments with slices were carried out in the presence of 1 μM mepyramine and 10 μM ranitidine. In the presence of 30 mM K^+, 1 μM mepyramine and 10 μM ranitidine, the release of tritium over spontaneous efflux represented 7.6 ± 0.4% of the total ($n = 3$ experiments) and was not significantly inhibited (by 12 ± 2%) in the presence of 2 μM (R)α-methylhistamine.

Thioperamide at increasing concentrations progressively antagonized the inhibitory effect of (R)α-methylhistamine with an IC_{50} value of 0.9 ± 0.6 μM (Figure 2), leading to a mean apparent K_i value of 22 ± 15 nM. Thioperamide, in high concentrations (10–100 μM), tended to enhance by about 20% the K^+-evoked release of tritium over the non-inhibited level (100%, Figure 2). Phenylbutanoylhistamine (200 μM) failed to antagonize the inhibitory effect of (R)α-methylhistamine but, when added alone in the presence of 20 mM K^+, 1 μM mepyramine and 10 μM ranitidine, significantly inhibited the K^+-evoked release of tritium by 44% (Table 1).

Release from synaptosomes

The basal tritium efflux from synaptosomes into the medium, evaluated in the presence of 2 mM K^+ represented 6290 ±

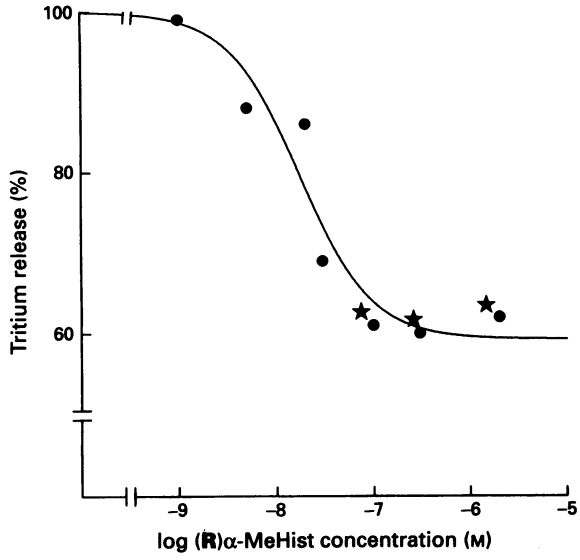

Figure 1 Effect of (R)α-methylhistamine ((R)α-MeHist) on K^+-evoked tritium release from slices of rat entorhinal cortex. Slices were allowed to synthesize [^{3}H]-acetylcholine during a 45 min preincubation in the presence of [^{3}H]-choline. After several washing steps, slices were distributed into tubes, incubated for 5 min in the presence of mepyramine (1 μM) and ranitidine (10 μM) with or without (R)α-MeHist, and subsequently incubated for 2 min in the presence of 2 or 20 mM K^+ (final concentration). The basal tritium efflux (2 mM K^+) represented 9.0 ± 0.5% of total tritium per 2 min and was not significantly modified in the presence of (R)α-MeHist. The release of tritium induced by 20 mM K^+ was calculated in each experiment as the difference between mean tritium levels in 20 and 2 mM K^+ media, respectively, and represented, in the absence of (R)α-MeHist, 3.5 ± 0.4% of the total (tissue plus medium) tritium. Results are expressed as percentages of this value. Each point represents the results from three separate experiments with 3–4 determinations. *$P < 0.05$ as compared with 20 mM K^+ in the absence of (R)α-MeHist.

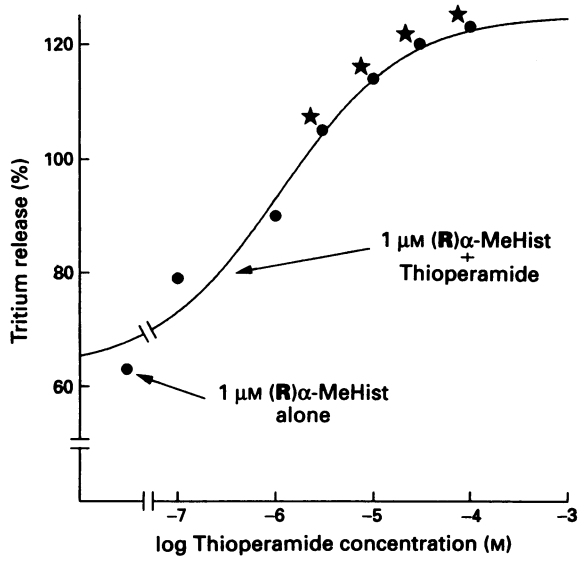

Figure 2 Effect of thioperamide on the inhibition by (R)α-methylhistamine ((R)α-MeHist) of the K^+-evoked tritium release from slices of rat entorhinal cortex. The spontaneous efflux of tritium (2 mM K^+) represented 9.5 ± 0.5% of total per 2 min, and tritium release induced by 20 mM K^+ over spontaneous efflux (expressed as a percentage of total (tissue plus medium) tritium) represented 5.8 ± 0.6%. Results are expressed as a percentage of this value. Thioperamide in increasing concentrations was added, in the presence of 1 μM mepyramine and 10 μM ranitidine, to (R)α-MeHist in a fixed concentration (1 μM) which, alone, inhibited the K^+-evoked tritium release from slices by 37%. Each point represents the results from three separate experiments with 4 determinations. *$P<0.05$ as compared with 1 μM (R)α-MeHist alone.

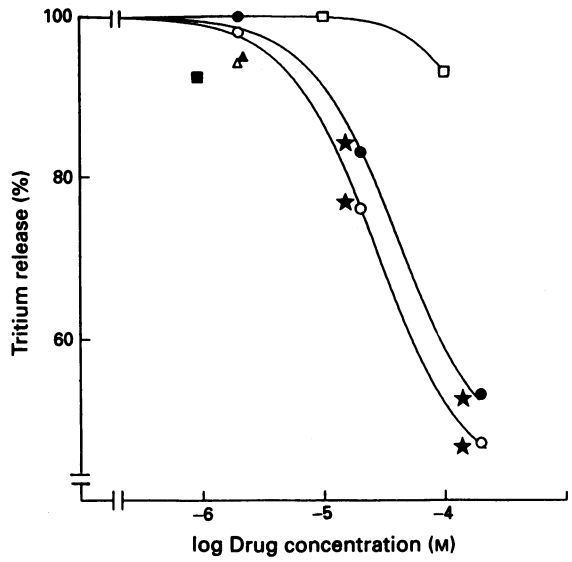

Figure 3 Modulation by various imidazole derivatives of tritium release from synaptosomes of rat entorhinal cortex depolarized with 10 mM K^+: (□) histamine; (●) phenylbutanoylhistamine; (○) phenylpropylhistamine; (■) imetit; (△) (R)αmethylhistamine; (▲) thioperamide. The spontaneous efflux of tritium (2 mM K^+) represented 10.0 ± 0.8% of total tritium per 2 min. In the absence of added agents, the release of tritium induced over basal efflux by incubation for 2 min with 10 mM K^+ represented 4.4 ± 0.6% of the total. Results are expressed as percentages of this value. Each point represents mean results from two separate experiments with quadruplicate determinations. Similar results were obtained in the presence of 1 μM mepyramine and 10 μM ranitidine. *$P<0.05$ as compared with 10 mM K^+ alone.

Table 1 Influence of (R)α-methylhistamine and phenylbutanoylhistamine on K^+-induced tritium release from slices and synaptosomes of rat entorhinal cortex

	% tritium release	
	Slices	*Synaptosomes*
Control	5.8 ± 0.6	5.4 ± 0.3
(R)α-methylhistamine (2 μM)	3.7 ± 0.6* (−36%)	5.0 ± 0.4 (−7%)
Phenylbutanoylhistamine (200 μM)	3.2 ± 0.5* (−44%)	3.1 ± 0.3** (−43%)

Slices and synaptosomes were depolarized for 2 min in the presence of 1 μM mepyramine, 10 μM ranitidine and either 20 mM K^+ (slices) or 10 mM K^+ (synaptosomes) (final concentration), respectively. Values represent the K^+-induced tritium release over spontaneous efflux, i.e. the difference between mean tritium levels in 20 mM K^+ (slices) or 10 mM K^+ (synaptosomes) and 2 mM K^+ media, respectively, expressed as a percentage of total (tissue plus medium) tritium. The spontaneous efflux (2 mM K^+) represented 9.1 ± 0.3% (slices) and 13.8 ± 0.2% (synaptosomes) of total tritium, per 2 min. Values in parentheses indicate percentage changes as compared with 10 mM K^+ or 20 mM K^+ alone (controls). Values are the mean ± s.e.mean of 3–9 determinations in two separate experiments. *$P<0.05$; **$P<0.01$ as compared with respective controls.

448 d.p.m. mg^{-1} protein, i.e. 13.0 ± 0.8% of the total tritium (present in tissue plus medium), per 2 min. The release elicited by 10 mM K^+ over basal efflux corresponded to 5.0 ± 0.3% of the total ($n = 11$ experiments) (Table 1 and Figure 3) and was not significantly modified in the presence of 1 μM imetit, 2 μM (R)α-methylhistamine, 100 μM histamine or 2 μM thioperamide tested in the absence (Figure 3) or presence (Table 1 and data not shown) of 1 μM mepyramine and 10 μM ranitidine. It was significantly ($P<0.05$) reduced by 32 ± 6% ($n = 3$ experiments) in the presence of 200 μM oxotremorine and was inhibited in a concentration-dependent manner by phenylbutanoylhistamine and phenylpropylhistamine (Figure 3). The inhibition by 46 ± 4% ($n = 7$ experiments) observed in the presence of 200 μM phenylbutanoylhistamine was not significantly diminished in the presence of 1 mM histamine, 1 μM mepyramine, 10 μM ranitidine, 200 μM burimamide, 60 μM impromidine, 2 μM thioperamide or 1 μM scopolamine (not shown). It was neither mimicked nor antagonized in the presence of 0.1 μM VIP or TRH (not shown). The inhibitory effect of 200 μM phenylbutanoylhistamine was less marked (−19 ± 1%, $n = 3$ experiments), although still significant ($P<0.05$), in the presence of 20 mM K^+. Although of limited magnitude, a reproducible and significant ($P<0.01$) decrease (by 11 ± 1%) of the basal tritium efflux was observed after a 2 min incubation of synaptosomes with 200 μM phenylbutanoylhistamine or phenylpropylhistamine in 2 mM K^+ ($n = 5$ experiments).

Discussion

It was recently reported that histamine H_3 receptors modulate the release of [^{3}H]-acetylcholine from superfused slices of entorhinal cortex (Clapham & Kilpatrick, 1992). The present data, obtained in a non-superfused slice system (Arrang *et al.*, 1983), confirm this observation. The tritium release from depolarized slices, previously shown to consist mainly of [^{3}H]-acetylcholine (Barnes *et al.*, 1989), was potently inhibited by (R)α-methylhistamine, a selective H_3-receptor agonist (Figure 1). The response to (R)α-methylhistamine was inhibited by the selective H_3-receptor antagonist thioperamide (Figure 2), with a mean apparent K_i value (22 ± 15 nM) not significantly different from its K_i values at H_3 receptors modulating [^{3}H]-histamine release from brain slices ($K_i = 5.6 \pm 1.0$ nM, Arrang *et al.*, 1987a) or synaptosomes ($K_i = 5.6 \pm 1.4$ nM, Garbarg *et al.*, 1992). In

contrast, its potency was increased approximately 10 fold in the presence of the H_1- and H_2-receptor antagonists, mepyramine (1 μM) and ranitidine (10 μM). Although both drugs did not significantly affect the K^+-evoked release, Clapham & Kilpatrick (1992) postulated from this result that H_1 and H_2 receptor stimulation might enhance acetylcholine release. Such a stimulation might also occur *in vivo* since it was recently shown by microdialysis that endogenous histamine stimulates endogenous acetylcholine release in rat hippocampus, mainly via interaction with H_2 receptors (Mochizuki *et al.*, 1994). It has also been suggested that stimulation of H_2 receptors releases endogenous noradrenaline in rat hypothalamus (Blandina *et al.*, 1989) and endogenous enkephalin in mouse striatum (Garbarg *et al.*, 1991).

In synaptosomes of entorhinal cortex, however, tritium release remained unaltered in the presence of two H_3-receptor agonists (Table 1 and Figure 3). Previous studies performed on the regulation of histamine release by presynaptic H_3-autoreceptors (Arrang *et al.*, 1985) or galanin receptors (Arrang *et al.*, 1991) and using very similar experimental conditions have shown that the maximal inhibitory effect observed in synaptosomes was smaller than in slices. However, although it cannot be entirely ruled out, the existence of a small modulation in synaptosomes seems to be unlikely since it was not observed in conditions (10 mM K^+) previously shown to increase markedly the maximal inhibitory effect of galanin on histamine release in rat hypothalamus and hippocampus (Arrang *et al.*, 1991). Moreover, the expected modulation by oxotremorine of tritium release from synaptosomes preloaded with [^{3}H]-choline (Raiteri *et al.*, 1984; Marchi & Raiteri, 1985) was found in the same experiments (not shown). Therefore, these data with synaptosomes strongly suggest that H_3 receptors modulating acetylcholine release in slices are not, for the most part, presynaptically located on cholinergic nerve terminals, but are found either on non-cholinergic nerve endings impinging on the former, or postsynaptically on intrinsic perikarya. The postsynaptic location of these H_3 receptors may account, at least partially, for the decrease in the number of H_3 receptors observed in cerebral cortex and striatum after local infusion of neurotoxins (Cumming *et al.*, 1991; Pollard *et al.*, 1993). Moreover, a physiological function of these receptors in the control of acetylcholine release is suggested by the participation of H_3 receptors in the endogenous modulation of acetylcholine release as shown by the facilitatory effect of thioperamide observed both *in vitro* (Figure 2, Clapham & Kilpatrick, 1992) and *in vivo* (Mochizuki *et al.*, 1994). However, the weak and relatively short-lasting effects of H_3 ligands observed *in vivo* on acetylcholine release as measured by microdialysis (Mochizuki *et al.*, 1994) suggest that this tonic modulation of acetylcholine release is probably minor, when compared to the modulation of histamine release (Arrang *et al.*, 1987a).

In contrast to thioperamide, phenylbutanoylhistamine, an H_3-receptor antagonist with moderate potency (Lipp *et al.*, 1988; 1992; Stark *et al.*, 1990; 1994) failed to antagonize the inhibitory effect of (R)α-methylhistamine on acetylcholine release from slices of rat entorhinal cortex (Clapham & Kilpatrick, 1992). In addition phenylbutanoylhistamine failed to antagonize the (R)α-methylhistamine-induced inhibition of non-adrenergic non-cholinergic contractions of the guinea-pig ileum (Taylor & Kilpatrick, 1992) and it was suggested from these data that this compound might discriminate several H_3-receptor subtypes. In order to reinvestigate this hypothesis, we further analysed the effects of phenylbutanoylhistamine as well as its amine derivative, phenylpropylhistamine, a compound with a ten times lower affinity at H_3 receptors (Lipp *et al.*, 1988; 1992; Stark *et al.*, 1990; 1994). Surprisingly, phenylbutanoylhistamine, when added alone, inhibited acetylcholine release from slices (Table 1) and both compounds inhibited, with a similar potency and to the same extent (by about 50% when added at a final concentration of 200 μM), acetylcholine release from synaptosomes (Figure 3). Although the inhibitory effect of both drugs was concentration-dependent, no plateau was reached and the EC_{50} values and maximal effect (which was probably higher than that observed in the presence of 200 μM of both drugs) could not be determined (Figure 3). However, their potency could be roughly evaluated to be in the micromolar range. This effect was not mediated by H_3 receptors, being neither mimicked by (R)α-methylhistamine or imetit in synaptosomes, nor blocked by three H_3-receptor antagonists added at a high concentration. It did not involve H_1 or H_2 receptors since it was not reversed by mepyramine or ranitidine. The involvement of a histamine receptor site, different from the known H_1, H_2 or H_3 receptors, was also ruled out since no inhibition was observed in synaptosomes in the presence of 100 μM histamine and no antagonism of the effect of phenylbutanoylhistamine was found in the presence of 1 mM histamine. The inhibitory effect was not mediated by muscarinic autoreceptors, being unchanged in the presence of a high concentration of scopolamine and receptors for VIP, a peptide colocalized with acetylcholine in one type of cholinergic innervation in the cortex (Eckenstein & Baughman, 1984) are probably not involved. Even whether both drugs act via interaction with receptors or via another mechanism is unclear. The small modulation of the basal tritium efflux from synaptosomes observed in their presence might suggest an unspecific mechanism. However, the partially depolarized state of synaptosomes incubated in the presence of 2 mM K^+ has been previously suggested to account for a receptor-mediated modulation observed under basal conditions (Arrang *et al.*, 1987b). Hence the mechanisms responsible for the inhibition by phenylbutanoylhistamine and phenylpropylhistamine remain to be identified. Whatever the mechanism, these properties of both drugs probably lead to a large underestimation of their moderate H_3-antagonist potency and do not allow any conclusion to be drawn as to the possible existence of H_3-receptor subtypes.

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