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Histamine H_3 receptors mediate inhibition of noradrenaline release from intestinal sympathetic nerves

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1 The present study investigates whether presynaptic histamine receptors regulate noradrenaline release from intestinal sympathetic nerves. The experiments were performed on longitudinal muscle-myenteric plexus preparations of guinea-pig ileum, preincubated with [^{3}H]-noradrenaline.

2 In the presence of rauwolscine, electrically-induced [^{3}H]-noradrenaline release was inhibited by histamine or R-α-methylhistamine, whereas it was unaffected by pyridylethylamine, impromidine, pyrilamine, cimetidine, thioperamide or clobenpropit. The inhibitory effects of histamine or R-α-methylhistamine were antagonized by thioperamide or clobenpropit, but not by pyrilamine or cimetidine. In the absence of rauwolscine, none of these drugs modified the release of [^{3}H]-noradrenaline.

3 The modulatory action of histamine was attenuated by pertussis toxin and abolished by N-ethylmaleimide. Tetraethylammonium or 4-aminopyridine enhanced the evoked tritium outflow and counteracted the inhibitory effect of histamine. However, the blocking effects of tetraethylammonium and 4-aminopyridine were no longer evident when their enhancing actions were compensated by reduction of Ca^{2+} concentration in the superfusion medium.

4 Histamine-induced inhibition of tritium output was enhanced by ω-conotoxin or low Ca^{2+} concentration, whereas it was not modified by nifedipine, forskolin, rolipram, phorbol myristate acetate, H7 or lavendustin A.

5 The present results indicate that presynaptic H_3 receptors, located on sympathetic nerve endings, mediate an inhibitory control on intestinal noradrenergic neurotransmission. It is suggested that these receptors are coupled to G_i/G_o proteins which modulate the activity of N-type Ca^{2+} channels through a direct link, thus reducing the availability of extracellular Ca^{2+} at the level of noradrenergic nerve terminals.

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Abbreviations: EDTA, ethylenediaminetetraacetic acid; H7, 1-(5-isoquinolinylsulphonyl)-2-methyl piperazine dihydrochloride; PMA, phorbol myristate acetate

Introduction

Histamine is an important endogenous mediator which is widely distributed throughout the gastrointestinal tract (Burks, 1994) and regulates a variety of digestive pathophysiological events, including gastric acid secretion (Schubert & Makhlouf, 1996), intestinal motility (Leurs *et al*., 1991), vasomotor responses (Beyak & Vanner, 1995), peptic ulcer disease (Pattichis & Louca, 1995), intestinal inflammatory responses and allergic reactions (Raithel *et al*., 1995). All these functions are currently ascribed to specific interactions of histamine with three distinct receptor subtypes, named H_1, H_2 and H_3 (Hill *et al*., 1997).

H_3 receptors, initially identified as presynaptic autoreceptors located on axon terminals at central histaminic neurons (Arrang *et al*., 1983), can be pharmacologically distinguished from H_1 and H_2 receptors by virtue of their high affinity for selective agonists and antagonists, such as R-α-methylhistamine, imetit, thioperamide and clobenpropit (Hill *et al*., 1997). The recent molecular cloning and structural analysis of cDNA encoding the human H_3 receptor have lent considerable support to the pharmacological classification of this receptor subtype (Lovenberg *et al*., 1999). Indeed, upon transfection with H_3 receptor cDNA, C6 glioma cells could competitively bind several H_3 ligands with high potencies, whereas they showed no affinity for known H_1 and H_2 selective antagonists, including mepyramine, diphenhydramine and ranitidine (Lovenberg *et al*., 1999).

At present, a large body of evidence indicates that H_3 receptors are not confined to histamine-containing neurons, but they may also act as presynaptic heteroreceptors, both in the central and peripheral nervous system, where they regulate the release of various neurotransmitters, such as acetylcholine, noradrenaline, dopamine, serotonin, glutamate and neuropeptides (Hill *et al*., 1997; Brown & Haas, 1999). As far as noradrenaline is concerned, prejunctional H_3 receptors involved in the negative modulation of noradrenergic neurotransmission have been identified in several mammalian tissues, including brain (Schlicker *et al*., 1992; Alves-Rodrigues *et al*., 1998), spinal cord (Celuch, 1995), heart (Imamura *et al*., 1995), and blood vessels (Ishikawa & Sperelakis, 1987; Molderings *et al*., 1992). However, presynaptic histamine receptors do not appear to be present on sympathetic nerves supplying the rabbit pulmonary artery and rat vena cava, whereas in other peripheral systems, such as for instance the canine saphenous vein and rabbit kidney, the noradrenergic neurotransmission seems to be regulated by H_1 and/or H_2 receptors (for references see: Molderings *et al*., 1992).

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In the gastrointestinal tract, after initial observations suggesting a role for prejunctional histamine receptors in the modulation of cholinergic neurotransmission and the presence of H_3 binding sites at intestinal level (Trzeciakowski, 1987), consistent evidence was provided that histamine H_3 receptors are significantly implicated in the regulation of several digestive functions (for review see: Bertaccini & Coruzzi, 1995). Indeed, H_3 receptors mediate inhibitory effects of histamine on gastric acid secretion (Soldani *et al*., 1993) as well as the contractile activity of duodenum, ileum, jejunum and colon (Hew *et al*., 1990; Coruzzi *et al*., 1991; Leurs *et al*., 1991). Most of these effects have been ascribed to a negative control exerted by presynaptic H_3 receptors on the release of acetylcholine and non-adrenergic non-cholinergic mediators from nerve endings of myenteric plexus (Poli *et al*., 1991; Taylor & Kilpatrick, 1992). In addition, H_3 receptors appear to control serotonin release and histamine synthesis at the level of enterochromaffin and enterochromaffin-like cells, respectively (Hollande *et al*., 1993; Schworer *et al*., 1994).

The sympathetic nervous system plays a prominent role in the regulation of secretory and motor functions throughout the gastrointestinal tract (McIntyre & Thompson, 1992). Moreover, there is both clinical and experimental evidence that sympathetic dysfunctions are implicated in the pathophysiology of several digestive orders (McIntyre & Thompson, 1992). Previous studies have shown that noradrenergic nerve terminals supplying the digestive tract are equipped with presynaptic heteroreceptors, including muscarinic, opioid and insulin receptors (Fuder & Muscholl, 1995; Cheng *et al*., 1997), the activation of which results in positive or negative changes of noradrenaline release. However, the possibility that prejunctional histamine receptors participate in the control of intestinal sympathetic neurotransmission has not yet been examined. Overall, the present study was undertaken in order to assess whether there are histamine receptors at noradrenergic axon terminals of guinea-pig ileum involved in the modulation of noradrenaline release.

Methods

Animals

Male albino guinea-pigs, 300–350 g body weight, were used throughout this study. The animals were fed standard laboratory chow and tap water *ad libitum* and were not used for at least 1 week after their delivery to the laboratory. They were housed, four in a cage, in temperature controlled rooms on a 12-h light cycle at 22–24°C and 50–60% humidity. Their care and handling were in accordance with the provisions of the European Community Council Directive 86-609, recognized and adopted by the Italian Government.

Preparations

At the time of the experiment, the whole ileum was excised from the small intestine with the exception of the distal 10 cm, and longitudinal muscle strips with myenteric plexus attached were prepared according to the method of Paton & Vizi (1969).

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

The measurement of [^{3}H]-noradrenaline release from isolated guinea-pig ileum was carried out according to the procedure previously described (Blandizzi *et al*., 1993), with minor modifications. Longitudinal muscle strips of ileum, weighing 60–120 mg, were incubated for 30 min in Krebs solution at 37°C, aerated with 95% O_2 + 5% CO_2 (preincubation period), and then loaded with 1-7,8-[^{3}H]-noradrenaline (3.5 μCi ml^{-1}) for 60 min in 2 ml of Krebs solution. The Krebs solution had the following composition (mM): NaCl 113, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, glucose 11.5, ascorbic acid 0.03, disodium EDTA 0.1 (pH 7.4 ± 0.1). The latter two compounds were included in the medium in order to prevent the oxidative decomposition of noradrenaline. At the end of the loading period, the ileal strips were washed five times with Krebs solution, transferred to another organ bath (5-ml capacity) filled with Krebs solution and superfused at a flow rate of 1 ml min^{-1} with Krebs solution at 37°C, aerated with 95% O_2 + 5% CO_2. Unless otherwise stated, the superfusing Krebs solution contained cocaine 5 μM and hydrocortisone 5 μM to prevent the reuptake of noradrenaline, and rauwolscine 1 μM, a selective α_2-adrenoceptor antagonist, to prevent activation of presynaptic α_2-adrenoceptors by endogenous noradrenaline. However, rauwolscine was omitted in some experiments. The first 60-min collection of effluent was discarded (preperfusion), after which 3-min fractions were collected for 90 min. During the superfusion period, the ileal preparations were subjected to electrical field stimulation, delivered as square wave pulses (10 V cm^{-1}) of 0.5 ms duration at 8 Hz (480 pulses) in the 3rd (S_1) and 20th (S_2) collection periods. These stimulation parameters led to a consistent [^{3}H]-noradrenaline release. However, it can not be excluded that a concomitant release of other neurotransmitters, such as acetylcholine and neuropeptides, might have occurred also upon electrical stimulation of ileal tissues at the frequency of 8 Hz. At the end of superfusion, the radioactivity of fractions was determined by liquid scintillation counting (Betamatic, Kontron Instruments, Milan, Italy), and the radioactive content of ileal strips was also measured. For this purpose, each preparation was weighed and then incubated in 1 ml of 10% trichloracetic acid at room temperature for 30 min. An aliquot of the supernatant (50 μl) was added to 5 ml of scintillator and the tritium content of the tissue was measured by liquid scintillation spectrometry.

Experimental procedures

In the first series of experiments, the mechanisms underlying the increase in tritium outflow induced by electrical field stimulation were examined. For this purpose, the ileal preparations were exposed to tetrodotoxin, ω-conotoxin, a selective blocker of N-type Ca^{2+} channels (Dolphin, 1995), or low Ca^{2+} concentrations in the superfusion medium, from the 12th collection period onward.

In the second set of experiments the effects of histamine, histamine receptor agonists or histamine receptor antagonists on tritium efflux were assessed. Histamine and histamine receptor agonists were added to the superfusing Krebs solution in the 12th collection period. Histamine receptor antagonists were also added to the medium in the 12th fraction when tested alone, but they were present from the beginning of superfusion in agonist-antagonist interaction experiments. Exposure to each drug continued until the end of experiment.

The third group of experiments was performed in order to assay the effects of histamine, histamine receptor agonists and antagonists on ileal strips, superfused with Krebs solution where rauwolscine was omitted throughout the superfusion period. The time schedule for the exposure of ileal preparations to test drugs was as reported for the second experimental series.

The fourth experimental series was aimed to examine the effects of histamine on tritium efflux after blockade of regulatory G proteins. For this purpose, ileal preparations were exposed to pertussis toxin or N-ethylmaleimide, two pharmacological agents able to block G_i/G_o proteins through the ADP-ribosylation or alkylation of their sulfhydryl groups, respectively (Asano & Ogasawara, 1986; Dolphin, 1995). Some ileal strips were incubated with pertussis toxin for 6 h and then washed repeatedly with fresh Krebs solution for at least 1 h before loading with [^{3}H]-noradrenaline. In separate experiments, the ileal tissues were incubated with N-ethylmaleimide for 45 min, either in the absence or in the presence of dithiothreitol, which prevents the alkylation of sulfhydryl groups (Schlicker *et al*., 1994). The preparations were then washed at least five times with fresh Krebs solution before loading with [^{3}H]-noradrenaline. In both cases, the ileal tissues were challenged with histamine from the 12th collection period onward.

In the fifth set of experiments, histamine was tested on preparations superfused with Krebs solution containing tetraethylammonium or 4-aminopyridine in order to induce a blockade of voltage-dependent K^+ channels (Pongs, 1992). Tetraethylammonium or 4-aminopyridine were present in the medium, either alone or in combination with reduced concentration of Ca^{2+}, from the 9th collection period onward, whereas histamine was added to the superfusion medium from the 12th fraction onward. In all these experiments, after 60-min preperfusion, 3-min fractions of effluent were collected for 120 min and S_2 was delivered to the ileal tissues in the 30th collection period, since simultaneous reduction of the Ca^{2+} concentration in the medium and addition of tetraethylammonium or 4-aminopyridine caused an increase in basal tritium efflux if the interval elapsing until application of S_2 was not long enough.

The sixth group of experiments was designed in order to assess whether extracellular Ca^{2+} may play a role in the effects exerted by histamine on tritium efflux. For this purpose, ileal preparations were superfused, from the 9th collection period onward, with Krebs solution containing ω-conotoxin, nifedipine an antagonist of L-type Ca^{2+} channels (Dolphin, 1995), or decreasing Ca^{2+} concentrations. Histamine was present in the superfusion medium from the 12th fraction onward.

In the seventh series, to evaluate the possibility that cyclic AMP, serine/threonine protein kinases or tyrosine kinase pathways can be implicated in the effects of histamine on tritium outflow, groups of ileal preparations were exposed, in separate experiments, to the following drugs: forskolin, an activator of adenylyl cyclase (Daly *et al*., 1982), rolipram, a selective blocker of type-IV phosphodiesterase (Müller *et al*., 1996), phorbol myristate acetate (PMA), an activator of protein kinase C (Swartz, 1993), H7, an inhibitor of serine/threonine protein kinases (Brown & Haas, 1999), and lavendustin A, a tyrosine kinase blocker (O'Dell *et al*., 1991). Each of these drugs was added to the medium at the beginning of superfusion and remained in the superfusate until the end of the experiment. In all cases, histamine was present in the superfusion medium from the 12th fraction onward.

Calculations

Tritium efflux into the superfusate was calculated as the fraction of tritium present in the ileal strip at the onset of the respective collection period (fractional rate; min^{-1}). The increase in tritium outflow evoked by electrical stimulation was calculated as previously described (Colucci *et al*., 1998). When test drugs were added to the superfusion medium between S_1 and S_2, their effects on the evoked tritium outflow were expressed as ratio of the percentage release during S_2 over that obtained during S_1 (S_2/S_1). For quantification of the effect of pertussis toxin, N-ethylmaleimide, dithiothreitol, forskolin, rolipram, PMA, H7 and lavendustin A, the percentage release obtained during S_1 was used.

Potencies of histamine and histamine receptor agonists were expressed as EC_{50} (concentration of the agonist that produces 50% of the maximal response for that agonist); the per cent maximum inhibition of tritium outflow evoked by electrical stimulation in control experiments (E_{max}) was also evaluated. Antagonist potencies were expressed as pK_d values from the equation:

$$K_d = [B]/(DR - 1)$$

where B is the molar concentration of the antagonist and DR is the ratio of equally effective concentrations of the agonist (EC_{50}) in the presence and absence of the antagonist (Furchgott, 1972).

Drugs

The following drugs were used: 1-7,8-[^{3}H]-noradrenaline (specific activity: 36 Ci $mmol^{-1}$; Amersham Laboratories, Des Plaines, IL, U.S.A.); disodium hydrocortisone 21-phosphate, cocaine hydrochloride, tetrodotoxin, histamine dihydrochloride, pyrilamine maleate, N-ethylmaleimide, (−)-dithiothreitol, tetraethylammonium chloride, 4-aminopyridine, ω-conotoxin GVIA, nifedipine, pertussis toxin, forskolin, rolipram, H7 [1-(5-isoquinolinylsulphonyl)-2-methyl piperazine dihydrochloride] (Sigma Chemicals Co., St. Louis, MI, U.S.A.); rauwolscine hydrochloride; R-(−)-α-methylhistamine; phorbol 12-myristate 13-acetate (Research Biomedicals International, Natick, MA, U.S.A.); thioperamide maleate (Tocris Cookson, Bristol, U.K.); cimetidine (Italfarmaco, Milan, Italy); clobenpropit dihydrobromide (synthesized by Professor H. Timmerman, Vrije Universiteit, Amsterdam, The Netherlands); 2-pyridylethylamine, impromidine trihydrochloride (kindly provided by Smith Kline Beecham, King of Prussia, PA, U.S.A.); lavendustin A (Alexis Biochemicals, Läufelfingen, Switzerland). Other reagents were of analytical grade. Pertussis toxin was dissolved in 0.1 M sodium phosphate buffer (pH 7) with 0.5 M sodium chloride. Thioperamide maleate, forskolin and lavendustin A were dissolved in dimethylsulfoxide and further dilutions were made with distilled water; the final concentration of dimethylsulfoxide in the perfusion medium (0.1%) had no effects on the basal or evoked tritium outflow.

Statistical analysis

Results are given as mean ± s.e.mean. The significance of differences was evaluated by Student's *t*-test. When more than one group was compared with a control, significance was assessed by one way analysis of variance followed by Dunnett or Student-Newman-Keuls test. *P* values lower than 0.05 were considered significant; *n* indicates the number of experiments. The ileal preparations included in each test group were obtained from distinct animals, and therefore, in the present study, *n* refers also to the number of animals used per experimental group. EC_{50} values were interpolated from concentration-response curves. All statistical procedures and curve fitting were performed by means of personal computer programs.

Results

Effects of tetrodotoxin, ω-conotoxin or low Ca^{2+} concentrations in the superfusion medium

In control experiments ($n=10$), after a 60-min initial preperfusion period, the spontaneous tritium overflow approached a rate of 0.001 ± 0.00008 min^{-1} and did not vary significantly throughout the experiments. When the superfused ileum strips were subjected to electrical field stimulation, the tritium efflux increased significantly from 0.0011 ± 0.00012 min^{-1} to 0.0075 ± 0.0007 min^{-1} ($P<0.05$). The increase in tritium outflow evoked by electrical stimulation was observed usually in four consecutive 3-min fractions; the release reached a peak during this time and then declined exponentially to the prestimulation value. The evoked tritium efflux was $5.34\pm0.59\%$ for S_1 and $5.15\pm0.56\%$ for S_2, not significantly different from each other; the calculated ratio S_2/S_1 was 0.96 ± 0.05 (Figure 1A). Under these conditions, the tritium outflow induced by electrical stimulation was significantly decreased by tetrodotoxin (1 μM) or ω-conotoxin (0.001–0.01 μM) as well as by exposure of ileal preparations to low Ca^{2+} concentrations (from 2.5 to 0.6 mM) in the superfusion medium (Figure 2). Neither the pharmacological agents nor lowering of Ca^{2+} concentration modified the resting overflow of tritium (not shown).

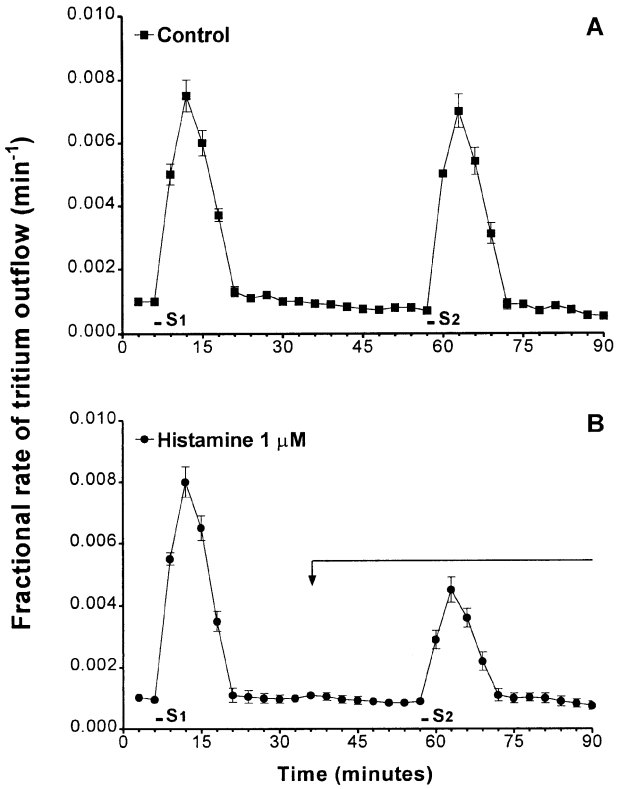

Figure 1 Tritium efflux from guinea-pig ileum longitudinal muscle strips preincubatd with [^{3}H]-noradrenaline. Abscissa: time of superfusate collection. Ordinate: efflux of tritium per min, expressed as fraction of tissue tritium at the onset of the respective collection period. Electrical field stimulation during S_1 and S_2 consisted of 480 pulses (10 V/cm, 0.5 ms) at 8 Hz. (A) Control experiments. (B) Effect of histamine 1 μM present in the superfusion medium during the time indicated by the horizontal bar. Each point represents the mean of 6–10 experiments ± s.e.mean (vertical bars).

Effects of histamine, histamine receptor agonists and histamine receptor antagonists

In the presence of α_2-adrenoceptor blockade by rauwolscine (1 μM), histamine, applied at the concentration of 1 μM, inhibited the evoked tritium outflow by 48.9% (Figure 1B; Table 1). The inhibitory effect of histamine could not be reproduced by pyridylethylamine (H_1 receptor agonist) or impromidine (H_2 receptor agonist), whereas it was mimicked by R-α-methylhistamine (H_3 receptor agonist) (Table 1). Under the same experimental conditions, the histamine receptor antagonists pyrilamine (H_1), cimetidine (H_2), thioperamide (H_3) or clobenpropit (H_3) did not significantly modify the electrically induced tritium output (Table 1).

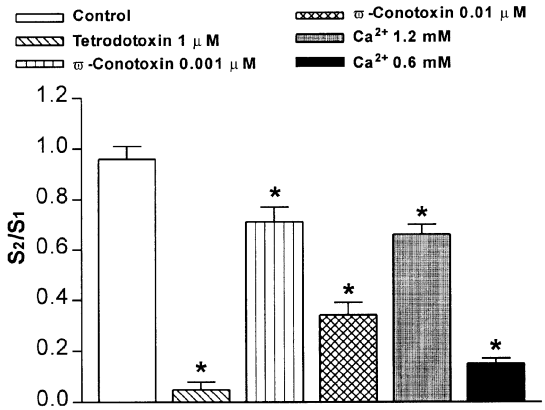

Figure 2 Effects of tetrodotoxin (1 μM), ω-conotoxin (0.001–0.01 μM), and reduction of Ca^{2+} concentration in the superfusion medium from 2.5 to 1.2 or 0.6 mM on tritium outflow evoked by electrical field stimulation of guinea-pig ileum longitudinal muscle strips preincubated with [^{3}H]-noradrenaline. Each column represents the mean of 5–7 experiments ± s.e.mean (vertical bars). Significant difference from control values: $^*P<0.05$.

Table 1 Effects of histamine, histamine receptor agonists and histamine receptor antagonists on the electricity evoked tritium outflow

	Electricity evoked tritium overflow (S_2/S_1) Rauwolscine (μM)	
	0	*1*
Control	1.02 ± 0.08	0.96 ± 0.05
Histamine 1 μM	0.97 ± 0.06	0.49 ± 0.03*†
Pyridylethylamine 1 μM	1.05 ± 0.07	0.98 ± 0.06
Impromidine 1μM	1.07 ± 0.06	1.01 ± 0.07
R-α-methyl-histamine 1 μM	0.99 ± 0.05	0.53 ± 0.04*†
Pyrilamine 1 μM	1.03 ± 0.06	0.94 ± 0.07
Cimetidine 1 μM	0.96 ± 0.08	0.97 ± 0.05
Thioperamide 1 μM	1.06 ± 0.07	1.03 ± 0.08
Clobenpropit 1 μM	1.01 ± 0.05	0.99 ± 0.06

Longitudinal muscle strips prepared from guinea-pig ileum were preincubated with [^{3}H]-horadrenaline, superfused either in the absence or in the presence of rauwolscine, and subjected twice to electrical stimulation. The effects of test drugs on tritium outflow evoked by electrical stimulation are expressed as S_2/S_1 values (ratio of the percentage release during the second stimulation over that obtained during the first stimulation). Each value represents the mean ± s.e.mean of 5–10 experiments. Significant difference from the control value: $^*P<0.05$; Significant difference from the corresponding value obtained in the absence of rauwolscine: †$P<0.05$.

When tested at increasing concentrations (from 0.0001 to 100 μM), both histamine and R-α-methylhistamine yielded inhibitory concentration-response curves, with EC_{50} values of 37.5 and 58.1 nM, and E_{max} values of 54.7 and 48.2%, respectively (Figure 3). The concentration-response curves obtained with histamine or R-α-methylhistamine were shifted to the right in the presence of thioperamide (1 μM) or clobenpropit (0.01 μM) (Figure 4). The following pK_d values were estimated for the antagonism of thioperamide and clobenpropit against histamine or R-α-methylhistamine: 8.25 (thioperamide vs histamine); 8.42 (thioperamide vs R-α-methylhistamine); 9.38 (clobenpropit vs histamine); 9.27 (clobenpropit vs R-α-methylhistamine). By contrast, pyrilamine (1 μM) or cimetidine (1 μM) did not modify the inhibitory effects of histamine and R-α-methylhistamine on the electrically induced tritium outflow (not shown). Histamine as well as all the histamine receptor agonists and antagonists tested did not affect the resting tritium outflow (not shown).

Effects of histamine, histamine receptor agonists and histamine receptor antagonists in the absence of rauwolscine

In control experiments ($n=8$) performed in the absence of α_2-adrenoceptor blockade, the tritium outflow evoked by S_1 was 1.82 ± 0.23% of tissue tritium content and the S_2/S_1 value accounted for 1.02 ± 0.08. Under these conditions, histamine, pyridylethylamine, impromidine, R-α-methylhistamine, pyrilamine, cimetidine, thioperamide or clobenpropit did not significantly modify the evoked tritium output elicited by electrical stimulation (Table 1). In addition, none of these drugs affected the basal tritium efflux (not shown).

Effects of histamine on longitudinal muscle strips of ileum pretreated with pertussis toxin or N-ethylmaleimide

Tritium outflow evoked by S_1 was not affected by pretreatment of ileal preparations with pertussis toxin (3 μg ml^{-1}), N-ethylmaleimide (30 μM), dithiothreitol (100 μM) or dithiothreitol plus N-ethylmaleimide ($S_1=5.49\pm0.76$, 5.56 ± 0.83, 5.27 ± 0.64, 5.39 ± 0.91% of tissue tritium content, respectively; $n=6$ for each treatment), and in all cases S_2/S_1 values did not differ significantly from those estimated in control experiments (Figure 5). The inhibitory effect of histamine (1 μM) on tritium overflow elicited by electrical stimulation was attenuated, but not abolished by pretreatment with pertussis toxin (Figure 5A), and it was completely prevented by N-ethylmaleimide (Figure 5B). Dithiothreitol did not interfere with the decreasing action of histamine on the evoked [^{3}H]-noradrena-

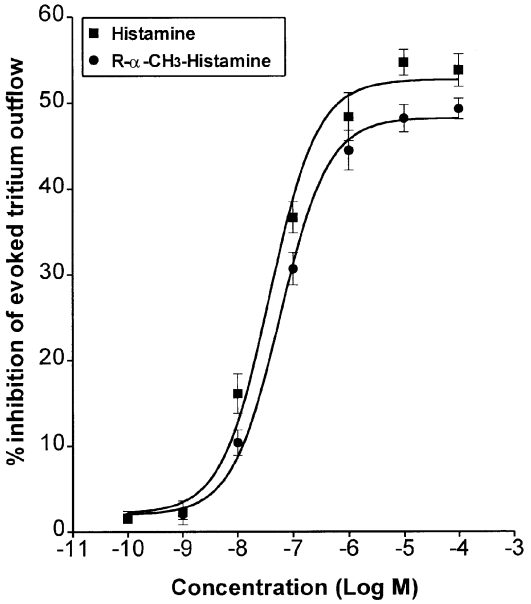

Figure 3 Effects of increasing concentrations of histamine or R-α-methylhistamine on tritium outflow evoked by electrical field stimulation of guinea-pig ileum longitudinal muscle strips preincubated with [^{3}H]-noradrenaline. Each point represents the mean of 4–6 experiments$\pm$s.e.mean (vertical bars).

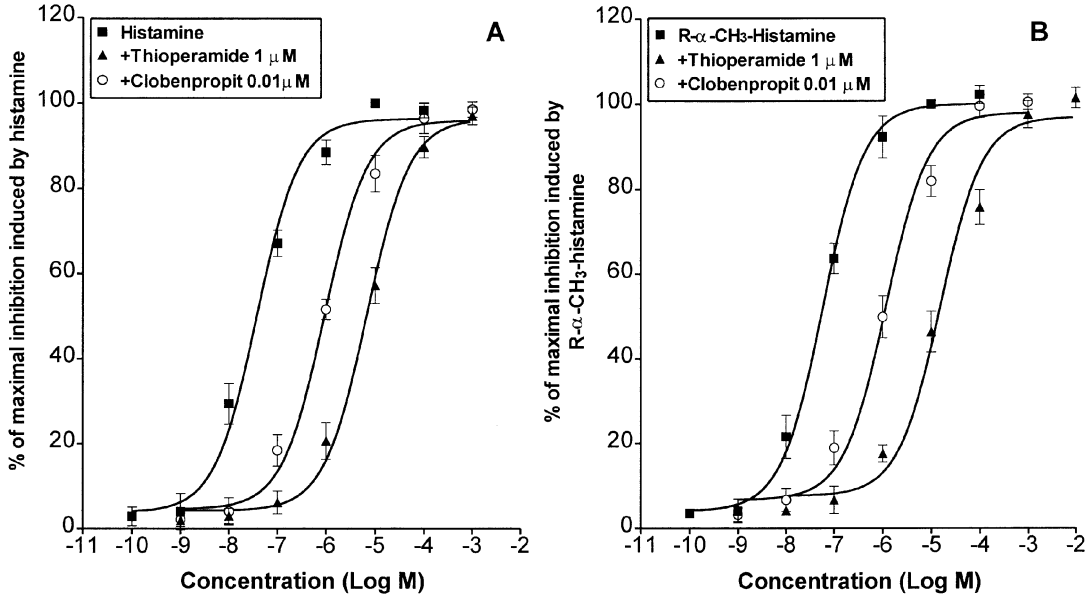

Figure 4 Effects of increasing concentrations of histamine (A) or R-α-methylhistamine (B), in the absence and in the presence of thioperamide (1 μM) or clobenpropit (0.01 μM), on tritium outflow evoked by electrical field stimulation of guinea-pig ileum longitudinal muscle strips preincubated with [^{3}H]-noradrenaline. Each point represents the mean of 4–6 experiments$\pm$s.e.mean (vertical bars).

line release, but totally counteracted the blocking action exerted by N-ethylmaleimide against the inhibitory effect of histamine (Figure 5B). The spontaneous outflow of radioactivity was not modified by pertussis toxin or dithiothreitol, whereas it was slightly increased by N-ethylmaleimide (not shown).

Effects of histamine in the presence of tetraethylammonium and 4-aminopyridine

Similar results were obtained in experiments designed to investigate the influence of tetraethylammonium or 4-aminopyridine on histamine-induced inhibition of [^{3}H]-noradrenaline release. When tested alone, both tetraethylammonium (1 mM) and 4-aminopyridine (1 mM) significantly enhanced the evoked tritium outflow (+44.9 and +84.1%, respectively), these effects being compensated by a reduction of Ca^{2+} concentration in the superfusion medium (from 2.5 to 1.2 or 0.6 mM, respectively) (Figure 6). The inhibitory effect of histamine (1 μM) on tritium efflux induced by electrical stimulation was significantly counteracted by tetraethylammonium or 4-aminopyridine in experiments where the superfusing solution contained 2.5 mM Ca^{2+} (Figure 6). However, the blocking actions exerted by tetraethylammonium and 4-aminopyridine against histamine were no longer evident when the ileal preparations were exposed to low Ca^{2+} concentration (1.2 and 0.6 mM, respectively) from the 9th collection fraction onward (Figure 6).

Effects of histamine in the presence of ω-conotoxin, nifedipine or reduced Ca^{2+} concentration in the superfusion medium

When tested at the concentration of 0.001 μM, ω-conotoxin reduced by 21.3% the increase in tritium efflux elicited by electrical stimulation and markedly enhanced the histamine-induced inhibition of the evoked tritium output (histamine alone: −43.6%; histamine plus ω-conotoxin: −84.3%) (Figure 7A). By contrast, nifedipine (0.01 μM) had no effect on the evoked tritium outflow and did not interfere with the inhibitory action of histamine (Figure 7A). Additional experiments showed that the extent of the inhibitory action exerted by histamine on the electrically-induced [^{3}H]-nora-

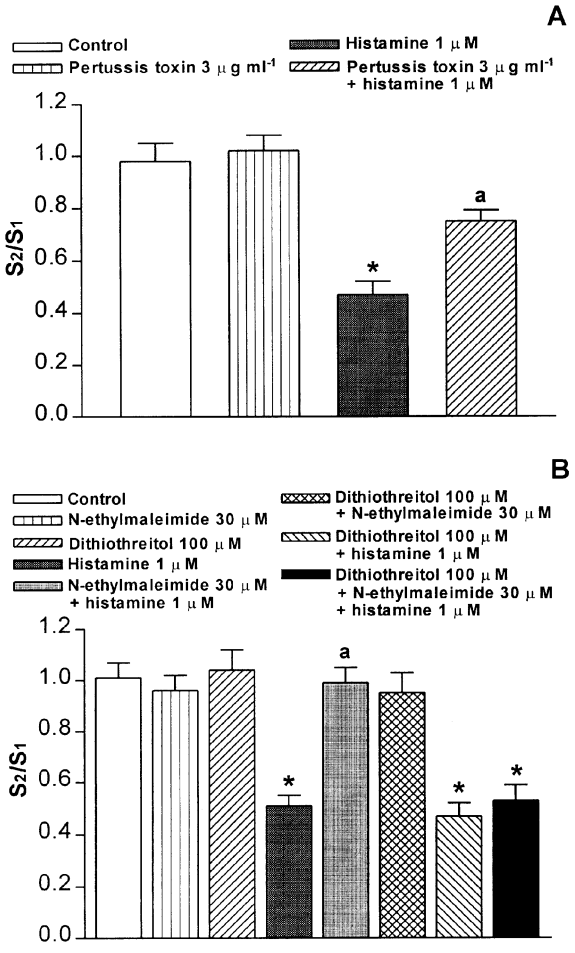

Figure 5 Effects of pertussis toxin (3 μg ml^{-1}) (A) and N-ethylmaleimide (30 μM), either alone or in the presence of dithiothrietol (100 μM) (B), on the inhibitory action exerted by histamine (1 μM) on the electrically evoked tritium outflow from guinea-pig ileum longitudinal muscle strips preincubated with [^{3}H]-noradrenaline. Each column represents the mean of 5–8 experiments ± s.e.mean (vertical bars). Significant difference from control values: $^*P<0.05$; significant difference from histamine alone: $^aP<0.05$.

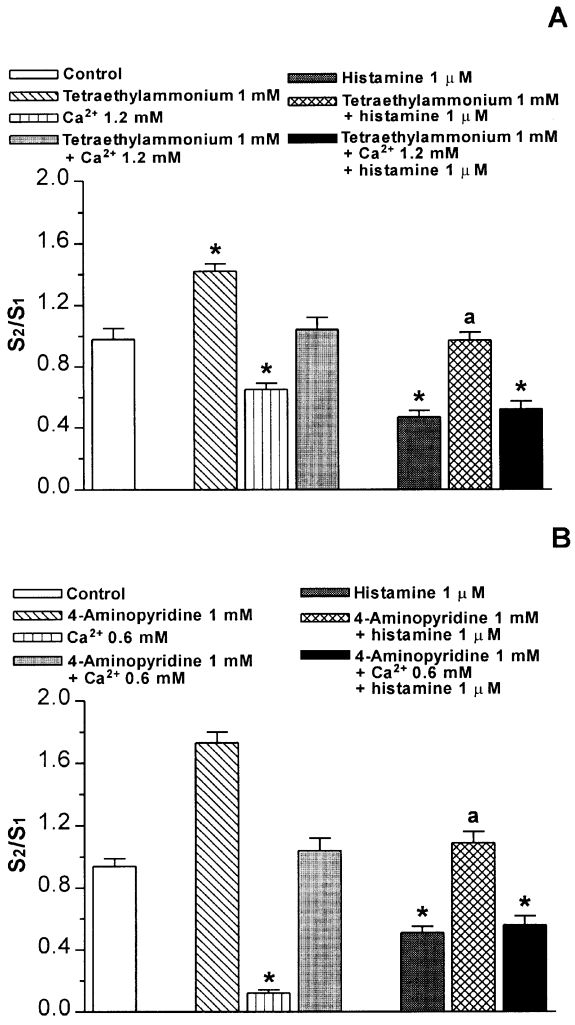

Figure 6 Effects of tetraethylammonium (1 mM), tested without or with reduction of Ca^{2+} concentration in the superfusion medium from 2.5 to 1.2 mM (A), and 4-aminopyridine (1 mM), tested without or with reduction of Ca^{2+} concentration in the superfusion medium from 2.5 to 0.6 mM (B), on the inhibitory action exerted by histamine (1 μM) on the electrically evoked tritium outflow from guinea-pig ileum longitudinal muscle strips preincubated with [^{3}H]-noradrenaline. Each column represents the mean of 5–8 experiments ± s.e.mean (vertical bars). Significant difference from control values: $^*P<0.05$; significant difference from histamine alone: $^aP<0.05$.

drenaline release increased as the Ca^{2+} concentration in the superfusing Krebs solution was lowered from the 9th collection period onward. In particular, the degree of histamine-induced inhibition of [^{3}H]-noradrenaline release accounted for −65.6% and −82.9% when the ileal preparations were exposed to 1.8 and 1.2 mM Ca^{2+}, respectively (Figure 7B). ω-Conotoxin, nifedipine and lowering of Ca^{2+} concentration in the superfusion medium did not significantly alter the resting tritium outflow (not shown).

Effects of histamine in the presence of forskolin, rolipram, PMA, H7 and lavendustin A

The tritium efflux induced by S_1 was not modified by exposure of ileal strips to rolipram (100 μM), PMA (1 μM), H7 (10 μM) or lavendustin A (10 μM), whereas it was increased by 45.7% in the presence of forskolin (10 μM) (Table 2). In all cases S_2/S_1 values were not significantly different from those obtained in control experiments (Table 2), and no relevant changes in the resting tritium output were detected (not shown). Moreover, none of these drugs was able to significantly interfere with the inhibitory action exerted by histamine (1 μM) on the electrically evoked release of radioactivity (Table 2).

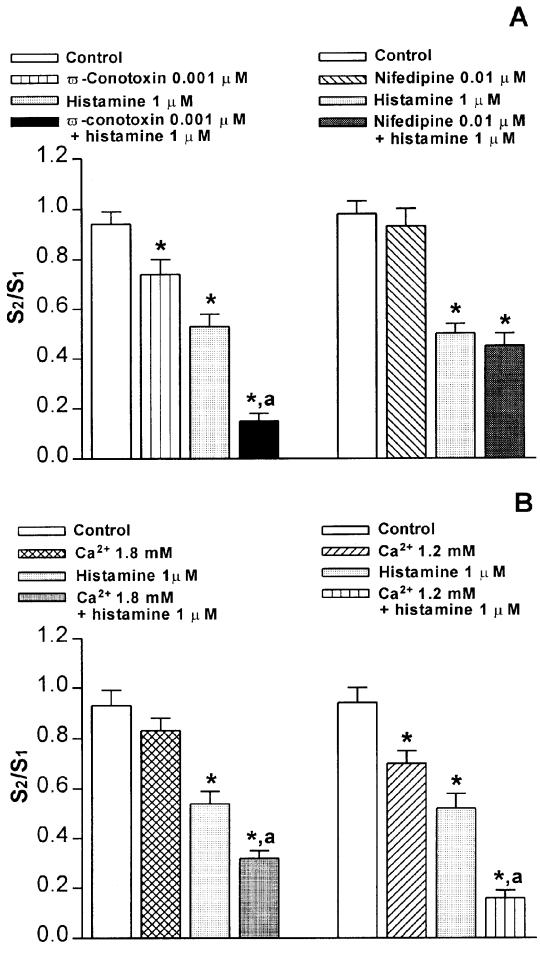

Figure 7 Effects of ω-conotoxin (0.001 μM), nifedipine (0.01 μM) (A), and reduction of Ca^{2+} concentration in the superfusion medium from 2.5 to 1.8 or 1.2 mM (B) on the inhibitory action exerted by histamine (1 μM) on the electrically evoked tritium outflow from guinea-pig ileum longitudinal muscle strips preincubated with [^{3}H]-noradrenaline. Each column represents the mean of 5–8 experiments ± s.e.mean (vertical bars). Significant difference from control values: $^*P<0.05$; significant difference from histamine alone: $^aP<0.05$.

Discussion

The main purpose of the present study was to pharmacologically identify and characterize presynaptic histamine receptors in the longitudinal muscle preparation of guinea-pig ileum. Accordingly, the present results demonstrate that sympathetic nerve terminals in small intestine are provided with functional H_3 receptors, the activation of which leads to a negative modulation of noradrenaline release evoked by electrical field stimulation, and extend the information available on the transduction pathways coupling prejunctional H_3 receptors to the intracellular effector systems in peripheral tissues. These findings may have some pathophysiological relevance, since when released at intestinal level during hypersensitivity reactions to allergens, histamine may mediate neuronal events (Wood, 1992).

In the present study, prior to addressing the question of whether histamine receptors regulate the sympathetic neurotransmission at digestive level, care was taken to verify that the measurement of electrically-induced outflow of radioactivity from ileal preparations may be taken as an index of endogenous noradrenaline release elicited by action potentials. In this regard, since in our experiments the electrically-evoked tritium efflux was Ca^{2+}-dependent and sensitive to both tetrodotoxin and ω-conotoxin, it has been assumed that, following the application of electrical stimuli, radiolabelled noradrenaline undergoes an exocitotic release operated by extracellular Ca^{2+} influx into adrenergic varicosities through N-type Ca^{2+} channels.

The pharmacological profile of presynaptic histamine receptors, characterized in the present study, conforms to the currently proposed criteria for the identification of H_3 receptors in functional experiments (Hill *et al*., 1997). Indeed, the histamine-induced inhibition of tritium outflow could be mimicked by R-α-methylhistamine, but not pyridylethylamine or impromidine. In addition, the inhibitory actions of both histamine and R-α-methylhistamine were antagonized with high potency by thioperamide or clobenpropit, whereas they were insensitive to selective antagonists for H_1 or H_2 receptors.

Table 2 Effects of forskolin, rolipram, PMA, H7 or lavendustin A on the inhibitory action of histamine on the electricity evoked tritium overflow

	Electricity evoked tritium overflow		
		S_2/S_1	
		Histamine (μM)	
	S_1	*0*	*1*
Control	5.14 ± 0.55	0.98 ± 0.06	0.51 ± 0.04†
Forskolin 100 μM	7.49 ± 0.65*	0.95 ± 0.07	0.46 ± 0.05†
Rolipram 100 μM	5.17 ± 0.61	1.02 ± 0.05	0.53 ± 0.06†
PMA 1 μM	5.63 ± 0.47	0.93 ± 0.06	0.49 ± 0.05†
H7 10 μM	5.81 ± 0.53	0.96 ± 0.08	0.56 ± 0.07†
Lavendustin A 10 μM	5.46 ± 0.07	1.03 ± 0.42	0.44 ± 0.04†

Longitudinal muscle strips prepared from guinea-pig ileum were preincubated with [^{3}H]-noradrenaline, superfused in the presence of rauwolscine, and subjected twice to electrical stimulation. The effects of test drugs on tritium outflow evoked by elecrical stimulation are expressed as S_1 (percentage of the tritium content of the tissue at the onset of the first electrical stimulation) or S_2/S_1 values (ratio of the percentage release during the second stimulation over that obtained during the first stimulation). Each value represents the mean ± s.e.mean of 5–7 experiments. Significant difference from the control value: $^*P<0.05$; significant difference from S_2/S_1 values obtained in the absence of histamine: †$P<0.05$.

These findings are in agreement with previous studies, dealing with presynaptic modulation of noradrenergic neurotransmission by H_3 receptors at both central and peripheral level (Fuder & Muscholl, 1995; Hill *et al*., 1997). For instance, the potency estimates obtained in the present study for thioperamide are comparable with those reported by other authors for [^{3}H]-noradrenaline release in guinea-pig brain, rat hippocampus and guinea-pig pulmonary artery (Rizzo *et al*., 1995; Alves-Rodrigues *et al*., 1998; Timm *et al*., 1998). It is also noteworthy that our estimated potencies for thioperamide and clobenpropit are quite consistent with values found for the same drugs in functional assays on guinea-pig intestinal preparations (Taylor & Kilpatrick, 1992; Bertaccini & Coruzzi, 1995).

West *et al*. (1990) have suggested the existence of multiple H_3 receptor subtypes, termed H_{3A} and H_{3B}, characterized by high and low affinity for thioperamide, respectively. However, this proposal is currently a matter of debate (Hill *et al*., 1997) and, at present, no conclusive evidence of functional H_{3B} receptors has been obtained in isolated tissues. Accordingly, although the issue of H_3 receptor heterogeneity was not specifically addressed in the present study, the potencies displayed by thioperamide against histamine and R-α-methylhistamine in our experiments would suggest that noradrenergic neurotransmission in guinea-pig ileum is modulated by H_3 receptors resembling the H_{3A} subtype.

It is interesting to note that in the present study the inhibitory effects of histamine and R-α-methylhistamine on [^{3}H]-noradrenaline release could be demonstrated only when ileal preparations were continuously exposed to the α_2-adrenoceptor antagonist rauwolscine. This observation is in line with data reported by other authors: in rat brain cortex and spinal cord as well as in pig retina, the activation of presynpatic H_3 receptors does not inhibit the evoked release of noradrenaline unless the superfusion medium contains phentolamine or rauwolscine (Schlicker *et al*., 1990; 1992; Celuch, 1995). Moreover, α_2-adrenoceptor blockade enhances the inhibitory effects of H_3 agonists in mouse brain (Schlicker *et al*., 1992). According to Schlicker *et al*. (1992), these findings reflect the occurrence of functional interactions between α_2-adrenoceptors and H_3 receptors, either at level of the receptors themselves or at steps involving the transduction pathways, which blunt or abolish the effect of histamine when the α_2-adrenoceptor-mediated inhibitory mechanism is activated. In our experiments, rauwolscine enhanced the electrically-induced tritium outflow, indicating that, in the absence of this drug, the released noradrenaline caused a consistent activation of presynaptic α_2-adrenoceptors. Therefore, since in the present study histamine and R-α-methylhistamine were not able to interfere with [^{3}H]-noradrenaline release in the absence of rauwolscine, a functional interaction between α_2-adrenoceptors and H_3 receptors, located on ileal noradrenergic nerves, is likely to occur. However, it must be noted also that the inhibitory effects of H_3 agonists on noradrenaline release were independent of α_2-adrenoceptor blockade in human saphenous vein and guinea-pig myocardium (Molderings *et al*., 1992; Endou *et al*., 1994), and therefore mutual interactions between α_2-adrenoceptors and H_3 receptors do not appear to occur ubiquitously in all tissues and species.

Signal transduction pathways used by H_3 receptors are currently under active investigation, and controversial findings have been reported on this point (Cherifi *et al*., 1992; Schlicker *et al*., 1994; Lovenberg *et al*., 1999). Therefore, in the present study efforts were made to highlight the mechanisms through which presynaptic H_3 receptors interact with intracellular effector systems at level of intestinal noradrenergic varicosities.

Binding studies, based on the use of stable analogues of GTP, have provided consistent evidence that H_3 receptors belong to the superfamily of G protein-coupled receptors (West *et al*., 1990; Hill *et al*., 1997). In keeping with this view, the present results, showing that the inhibitory action of histamine on noradrenaline release was attenuated by pertussis toxin and abolished by N-ethylmaleimide, suggest that the H_3 receptors, located on sympathetic nerve endings of guinea-pig ileum, are coupled to regulatory G proteins belonging to G_i/G_o subtypes. This finding was not totally unexpected, since in previous studies the participation of G_i/G_o proteins in the H_3 receptor-mediated modulation of noradrenergic neurotransmission was demonstrated by means of pretreatments with pertussis toxin and or N-ethylmaleimide, in mouse brain, rat spinal cord and guinea-pig heart (Endou *et al*., 1994; Schlicker *et al*., 1994; Celuch, 1995). In addition, inhibitory mechanisms of noradrenaline release involving the activation of G_i/G_o proteins have been previously reported for other presynaptic receptors, including α_2-adrenoceptors, opioid and adenosine receptors (for references see: Celuch, 1995).

In the case of G protein-linked presynaptic receptors, three major mechanisms have been suggested for the negative modulation of neurotransmitter release: (1) activation of presynaptic K^+ channels, resulting in a reduction of action potential efficacy; (2) inhibition of Ca^{2+} channels, leading to an impairment of the exocitotic machinery and (3) direct modulation of the vesicle release apparatus (Miller, 1998). In the present study, the blockade of K^+ channels by tetraethylammonium or 4-aminiopyridine both increased the evoked tritium outflow and counteracted the inhibitory action of histamine on [^{3}H]-noradrenaline release. However, the latter effect was no longer evident when the increment of tritium efflux, produced by tetraethylammonium or 4-aminopyridine, was compensated by a decrease in Ca^{2+} concentration in the superfusion medium. Therefore, since the blockade of K^+ channels is associated with an enhancement of Ca^{2+} influx into axon terminals, caused by a prolonged duration of action potential (Starke *et al*., 1989), our results suggest that the attenuation of histamine-induced inhibition of [^{3}H]-noradrenaline release by tetraethylammonium and 4-aminopyridine depends indirectly on an increased intraneuronal concentration of Ca^{2+}, whereas a coupling of H_3 receptors to K^+ channels appears to be less likely. Similar data have been obtained for prejunctional H_3 receptors modulating noradrenaline release in mouse brain cortex or glutamate release in rat dentate gyrus (Schlicker *et al*., 1994; Brown & Haas, 1999).

In the present study, at least two lines of evidence support the view that an inhibition of Ca^{2+} channels, with subsequent reduction of extracellular Ca^{2+} influx into the sympathetic nerve endings, may account for the H_3 receptor-mediated inhibition of evoked [^{3}H]-noradrenaline release from ileal preparations: (1) the inhibitory effect of histamine on the electrically-induced tritium outflow was markedly enhanced by ω-conotoxin, a selective blocker of N-type Ca^{2+} channels, which play a pivotal role in the regulation of exocitotic neurotransmitter release (Dolphin, 1995), whereas the blockade of L-type Ca^{2+} channels by nifedipine was without consequences, and (2) lowering of Ca^{2+} concentration in the superfusion medium resulted in a significant enhancement of histamine-induced inhibition of [^{3}H]-noradrenaline release. Overall, these findings, taken together with data reported from previous studies on mouse brain and guinea-pig heart (Endou *et al*., 1994; Schlicker *et al*., 1994), suggest that presynaptic H_3 receptors, once activated, inhibit N-type Ca^{2+} channels, causing a reduced availability of extracellular Ca^{2+} ions for stimulus-release coupling at the level of noradrenergic varicosities.

Very little is currently known about the intracellular signal transduction pathways through which H_3 receptors may modulate Ca^{2+} channels responsible for voltage-dependent release of noradrenaline. In general, receptors coupled to G_i/G_o proteins can regulate neuronal Ca^{2+} currents by interference with adenylyl cyclase, phospholipase C or tyrosine kinase pathways as well as by direct interaction with voltage-dependent Ca^{2+} channels (Dolphin, 1995; Diverse Pierluissi *et al*., 1997). In previous studies, histamine decreased forskolin-stimulated cyclic AMP formation in mouse L cells transfected with H_3 receptor cDNA (Lovenberg *et al*., 1999), but other authors failed to observe an inhibition of adenylyl cyclase activity in different tissues and cells (Cherifi *et al*., 1992; Schlicker *et al*., 1994). Moreover, Cherifi *et al*. (1992) described a negative coupling of H_3 receptors to phospholipase C in human HGT-1 cells. In this regard, it was speculated that H_3 receptor-mediated decrease in phosphoinositide turnover and diacylglycerol formation might decrease protein kinase C activity and, therefore, noradrenaline release from cardiac nerve endings (Imamura *et al*., 1995), but no evidence of protein kinase C involvement was obtained for H_3 receptors modulating glutamate release at central level (Brown & Haas, 1999). In the present study, various pharmacological tools were used in order to explore the possibility that the activation of presynaptic H_3 receptors interferes with the activity of adenylyl cyclase (forskolin, rolipram), serine-threonine protein kinases (PMA, H7) or tyrosine kinase (lavendustin A), but none of these drugs was able to modify the inhibitory effect of histamine on the evoked [^{3}H]-noradrenaline release. On these bases, since under most conditions the inhibition of Ca^{2+} channels by G_i/G_o proteins involves a direct action of the $\beta\gamma$ subunits of the G protein on the β subunit of voltage-dependent Ca^{2+} channels (Dolphin, 1998), it is conceivable that in our experimental model G_i/G_o proteins, coupled to H_3 receptors, inhibit Ca^{2+} influx into the noradrenergic varicosities through a direct interaction with N-type Ca^{2+} channels.

In conclusion, the present results suggest that histamine exerts an inhibitory control on intestinal noradrenergic neurotransmission through the activation of presynaptic H_3 receptors located on sympathetic nerve endings. According to our findings, it appears that these receptors are coupled to G_i/G_o proteins which modulate the activity of N-type Ca^{2+} channels through a direct link, thus causing a reduced availability of extracellular Ca^{2+} at level of ileal noradrenergic nerve terminals.

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