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Corrado Blandizzi · Rocchina Colucci
Martina Tognetti · Barbara De Paolis · Mario Del Tacca

H3 receptor-mediated inhibition of intestinal acetylcholine release: pharmacological characterization of signal transduction pathways

Abstract The present study investigates the mechanisms through which prejunctional histamine H_3 receptors modulate intestinal cholinergic neurotransmission. The experiments were performed on longitudinal muscle-myenteric plexus preparations of guinea pig ileum, preincubated with [^{3}H]choline, superfused with physiological salt solution containing hemicholinium-3, and subjected to electrical field stimulation. The stimulation-induced outflow of radioactivity was taken as an index of endogenous acetylcholine release.

The electrically induced [^{3}H]acetylcholine release was inhibited by histamine (EC_{50}=33.5 nM) or the H_3 receptor agonist R-α-methylhistamine (EC_{50}=41.6 nM), whereas it was not affected by pyridylethylamine (H_1 agonist), impromidine (H_2 agonist), pyrilamine (H_1 antagonist), cimetidine (H_2 antagonist), thioperamide or clobenpropit (H_3 antagonists). The inhibitory effects of histamine or R-α-methylhistamine were antagonized by thioperamide (pK_d= 8.31 and 8.53, respectively) or clobenpropit (pK_d=9.44 and 9.32, respectively), but not by pyrilamine or cimetidine. The modulatory action of histamine on the evoked tritium outflow was attenuated by pertussis toxin and abolished by N-ethylmaleimide, two selective blockers of G_i/G_o proteins. Tetraethylammonium or 4-aminopyridine, acting as inhibitors of voltage-dependent K^+ channels, enhanced the evoked tritium outflow when tested alone, and apparently counteracted the inhibitory effect of histamine. However, the blocking actions of tetraethylammonium and 4-aminopyridine were no longer evident when their enhancing actions were compensated by appropriate reductions of Ca^{2+} concentration in the superfusion medium. Histamine-induced inhibition of evoked tritium output was enhanced by ω-conotoxin, a selective blocker of N-type Ca^{2+} channels, or low Ca^{2+} concentration, whereas it was not modified by nifedipine, an antagonist of L-type Ca^{2+} channels. In addition, the inhibitory effect of histamine was not significantly affected by forskolin (activator of adenylyl cyclase), 8-bromo-cyclic AMP (a stable analog of cyclic AMP), rolipram (a selective blocker of type IV phosphodiesterase), phorbol myristate acetate (activator of protein kinase C), H-89 (N-(2-[p-bromocinnamyl-amino]ethyl)-5-isoquinolinesulfonamide, inhibitor of protein kinase A), Ro-31-8220 (2-{1-[3-(amidinothio)propyl]-1H-indol-3-yl}-3-(1-methylindol-3-yl)-maleimide, inhibitor of protein kinase C), KT5823 (N-methyl-(8R*,9S*,11S*)-(-)-9-methoxy-9-methoxycarbonyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1H,8H,11H-2,7b,11a-triazadibenzo [a,g]cycloocta[c,d,e]-trinden-1-one, inhibitor of protein kinase G), or lavendustin A (inhibitor of tyrosine kinase). The present results indicate that histamine inhibits intestinal cholinergic neurotransmission through presynaptic H_3 receptors coupled to G_i/G_o proteins. It is suggested that adenylyl cyclase, serine-threonine protein kinase and tyrosine kinase pathways are not implicated in this regulatory action, and that G_i/G_o proteins modulate the activity of N-type Ca^{2+} channels through a direct link, thus causing a reduced availability of extracellular Ca^{2+} at the level of ileal cholinergic nerve terminals.

Keywords Histamine H_3 receptors · Acetylcholine release · Enteric nervous system · Ileum · G proteins · Ca^{2+} channels · K^+ channels

C. Blandizzi · R. Colucci · M. Tognetti · B. De Paolis
M. Del Tacca (✉)
Division of Pharmacology and Chemotherapy,
Department of Oncology,
Transplants and Advanced Technologies in Medicine,
University of Pisa, Via Roma 55, 56126 Pisa, Italy
e-mail: m.deltacca@do.med.unipi.it,
Fax: +39-050-562020

Introduction

Histamine is an important endogenous mediator which regulates a variety of digestive pathophysiological events, including intestinal peptic ulcer disease (Pattichis and Louca 1995), motility (Leurs et al. 1991), intestinal inflammatory responses and allergic reactions (Raithel et al. 1995). All these functions are 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 can be 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 its pharmacological classification (Lovenberg et al. 1999). Indeed, upon transfection with H_3 receptor cDNA, C6 glioma cells could competitively bind several H_3 ligands, whereas they showed no affinity for known H_1 and H_2 antagonists (Lovenberg et al. 1999).

H_3 receptors may act as presynaptic heteroreceptors, both in the central and peripheral nervous system, where they regulate the release of various neurotransmitters (Hill et al. 1997). In particular, prejunctional H_3 receptors involved in the negative modulation of cholinergic neurotransmission have been identified in mammalian brain, airways and digestive system (Trzeciakowski 1987; Ichinose et al. 1989; Clapham and Kilpatrick 1992). In the gastrointestinal tract, after initial observations suggesting a role for prejunctional histamine receptors in the modulation of cholinergic neurotransmission (Trzeciakowski 1987), evidence was provided that histamine H_3 receptors are implicated in the regulation of several digestive functions (Bertaccini and Coruzzi 1995), including gastric acid secretion (Soldani et al. 1993) and the contractile activity of small and large intestine (Hew et al. 1990; 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 and Kilpatrick 1992).

In previous studies, the pharmacological profile of intestinal H_3 receptors has been characterized by indirect functional techniques, based on the effects of histamine receptor ligands on the contractile activity of smooth muscle elicited by electrical stimulation of myenteric neurons (Menkveld and Timmerman 1990; Bertaccini and Coruzzi 1995). Nevertheless, several aspects regarding the mechanisms through which the histaminergic pathways regulate the release of acetylcholine in the intestine remain to be elucidated. For instance, the electrically induced [³H]acetylcholine release was enhanced by the H_3 receptor antagonist thioperamide, suggesting the existence of a tonic inhibitory control by endogenous histamine on intestinal cholinergic transmission (Poli et al. 1991). However, this observation was not confirmed in functional experiments dealing with the assessment of electrically induced cholinergic motor responses (Schlicker et al. 1994a).

Little is currently known about the intracellular signal transduction pathways coupled to H_3 receptors at either presynaptic or postsynaptic locations (Bertaccini and Coruzzi 1995; Hill et al. 1997). Previous studies suggested that H_3 receptor activation reduces the entry of Ca^{2+} into cholinergic nerve endings of guinea pig duodenum (Poli et al. 1994), but the molecular mechanisms through which H_3 receptors interact with intracellular effector systems remain to be clarified. In addition, experiments performed on cell lines transfected with human or rat H_3 receptor cDNA indicated a negative coupling of these receptors with adenylyl cyclase in both species (Lovenberg et al. 2000). However, studies dealing with histamine-mediated modulation of noradrenergic neurotransmission failed to demonstrate an involvement of cyclic AMP in the signaling pathway of prejunctional H_3 receptors at both central and peripheral level (Schlicker et al. 1994b; Celuch 1995; Blandizzi et al. 2000).

The present study was designed to pursue a dual purpose: (1) to examine the mechanisms through which prejunctional histamine receptors modulate the release of acetylcholine from intestinal cholinergic nerves; (2) to gain further insight into the pharmacological profile of histamine receptors involved in the regulation of intestinal cholinergic neurotransmission. In order to assess the effects of drugs directly on cholinergic nerve endings, longitudinal muscle-myenteric plexus preparations of guinea pig ileum were incubated with [³H]choline, and the outflow of radioactivity elicited by electrical field stimulation was used as an index of endogenous acetylcholine release.

Materials and methods

Animals. Male albino guinea pigs (Harlan Italy, Udine, Italy), 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 as previously reported (Colucci et al. 1998).

Measurement of [³H]acetylcholine release. The measurement of [³H]acetylcholine release from isolated guinea pig ileum was carried out according to the procedure previously described (Colucci et al. 1998), with minor modifications. Longitudinal muscle strips of ileum, 3–4 cm long, 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 methyl-[³H]choline (3 µCi/ml) for 45 min in 3 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 (pH 7.4±0.1). Electrical pulses (0.1 ms duration) were applied for 30 min at a frequency of 1 Hz during the incubation period. 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), and superfused at a flow rate of 1 ml/min with Krebs solution at 37°C, aerated with 95% O_2 + 5% CO_2. The superfusing Krebs solution contained 3 µM hemicholinium-3 in order to inhibit the reuptake of [³H]choline generated by the hydrolysis of released [³H]acetylcholine. 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 voltage drop between the electrodes of each organ bath) of 1 ms duration at 1 Hz (180 pulses) in the 3rd (S_1) and 20th (S_2) collection periods. At the end of superfusion, the radioactivity of fractions was determined by liquid scintillation counting (Betamatic; Kontron Instruments, Milan, Italy), and the radioactive con-

tent of ileal strips was also measured. Each preparation was weighed, incubated in 1 ml of 10% trichloroacetic acid at room temperature for 30 min, homogenized, and then centrifuged (1000 g) at room temperature for 10 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 solution, from the 12th collection period onward.

In the second set of experiments the effects of histamine, histamine receptor agonists or histamine receptor antagonists on [³H]acetylcholine release were determined. Histamine or histamine receptor agonists were added to the superfusing Krebs solution in the 12th collection period. Histamine receptor antagonists were also added to the superfusion solution in the 12th collection period 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 the experiment.

The third group of experiments was designed 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 and 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 methyl-[³H]choline. In this series, ileal preparations, to be used as controls or to assay the effects of histamine alone on [³H]acetylcholine release, were maintained in the absence of pertussis toxin for 6 h and were then subjected to repeated washings for at least 1 h before incubation with methyl-[³H]choline. 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. The preparations were then washed at least five times with fresh Krebs solution before incubation with methyl-[³H]choline. In both cases, the ileal tissues were challenged with histamine from the 12th collection period onward.

In the fourth set of experiments, histamine was tested on ileal 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 solution, either alone or in combination with reduced concentration of Ca^{2+}, from the 9th collection period onward, whereas histamine was added to the superfusion solution from the 12th collection period 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 superfusing solution 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 fifth group of experiments was designed to assess whether extracellular Ca^{2+} plays a role in the effects exerted by histamine on [³H]acetylcholine release. 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 solution from the 12th collection period onward.

In the sixth series, we evaluated the possibility that adenylyl cyclase, serine/threonine protein kinase or tyrosine kinase pathways were involved in the effects of histamine on acetylcholine release. Groups of ileal preparations were exposed, in separate experiments, to the following drugs: forskolin, an activator of adenylyl cyclase

(Daly et al. 1982), 8-bromo-cyclic AMP (8-Br-cAMP), a stable analog of cyclic AMP (Chen et al. 1998), rolipram, a selective blocker of type IV phosphodiesterase (Müller et al. 1996), phorbol myristate acetate (PMA), an activator of protein kinase C (Swartz 1993), H-89, an inhibitor of protein kinase A (Hidaka and Kobayashi 1992), Ro-31-8220, an inhibitor of protein kinase C (Beltman et al. 1996), KT5823, an inhibitor of protein kinase G (Kase et al. 1987), and lavendustin A, a blocker of tyrosine kinase (O'Dell et al. 1991). Each drug was added to the solution at the beginning of superfusion and remained in the superfusate until the end of experiment. Histamine was present in the superfusion solution from the 12th collection period onward.

Calculations. The amount of radioactivity released during superfusion was dependent upon the amount of radioactivity accumulated by the strips. In order to correct for this, the quantity of released radioactivity had to be normalized. This was achieved by dividing radioactivity in the first fraction by the total radioactivity present in the ileal strip at the start of the collection period, namely, by the sum of the radioactivity found in all fractions plus that remaining in the strip at the end of the experiment. A similar procedure was used for determination of tritium efflux in the subsequent fractions. In these determinations, however, the denominator was reduced by the amounts released into the prior fractions. Thus, the outflow of tritium was calculated as fraction of the tritium content of 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 the difference of 'total tritium outflow during the four collection periods subsequent to the onset of stimulation' minus 'estimated basal outflow.' The basal outflow was estimated as mean value of the tritium efflux determined during four collection periods: two immediately before the onset of electrical stimulation and two subsequent to the 12-min period in which the tritium outflow was increased by electrical stimulation. The evoked outflow was then expressed as a percentage of the tritium content of the tissue at the onset of electrical stimulation (Colucci et al. 1998). When test drugs were added to the superfusion solution between S_1 and S_2, their effects on the evoked tritium outflow were expressed as a ratio of the percentage release during the second (S_2) and the first (S_1) stimulation (S_2/S_1), namely, in the presence and absence of drug. For quantification of the effects of pertussis toxin, *N*-ethylmaleimide, dithiothreitol, forskolin, 8-Br-cAMP, rolipram, PMA, H-89, Ro-31-8220, KT5823 and lavendustin A, the S_1 value was used.

Potencies of histamine and *R*-α-methylhistamine were expressed as EC_{50} (concentration of the agonist that produces 50% of the maximal response for the agonist); the percent 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:

$$K_d = [B]/(DR-1) \tag{1}$$

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: methyl-[³H]choline chloride (specific activity: 75 Ci/mmol; Amersham Laboratories, Des Plaines, Ill., USA); tetrodotoxin, hemicholinium-3 bromide, histamine dihydrochloride, pyrilamine maleate, *N*-ethylmaleimide, (-)-dithiothreitol, tetraethylammonium chloride, 4-aminopyridine, ω-conotoxin GVIA, nifedipine, pertussis toxin, forskolin, 8-bromo-cyclic AMP, rolipram, Ro-31-8220 (2-{1-[3-(amidinothio)propyl]-1*H*-indol-3-yl}-3-(1-methylindol-3-yl)-maleimide methanesulfonate), H-89 (*N*-(2-[*p*-bromocinnamylamino]ethyl)-5-isoquinolinesulfonamide hydrochloride) (Sigma Chemicals, St. Louis, Mo., USA); *R*-(-)-α-methylhistamine, phorbol 12-myristate 13-acetate (Research Biomedicals International, Natick, Mass., USA); thioperamide maleate (Tocris Cookson, Bristol, UK); cimetidine (Italfarmaco, Milan, Italy); KT5823 (*N*-methyl-(8*R**,9*S**,11*S**)-(-)-9-methoxy-9-methoxycarbonyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1*H*, 8*H*,11*H*-2,7b,11a-triazadibenzo[a,g]cycloocta[c,d,e]-trinden-1-one;

Biomol Research Laboratories, Plymouth Meeting, Pa., USA); 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., USA); 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 superfusion solution (0.1%) had no effects on the basal or evoked tritium outflow.

Statistical analysis. Results are given as means ± standard error of the mean (SEM). 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 (Graph-Pad Prism, software package version 2.01 for Windows 95, and In-Stat, software package version 2.01 for MacIntosh; both from GraphPad Software, San Diego, Calif., USA).

Results

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

In control experiments (*n*=12), after a 60-min initial preperfusion period, the spontaneous tritium overflow approached a rate of 0.0020 ± 0.00014 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 0.0019 ± 0.00012 min^{-1} to 0.0057 ± 0.00025 min^{-1} (*P*<0.05). The increase in [³H]acetylcholine release evoked by electrical stimulation was observed usually in four consecutive 3-min fractions; the release reached a peak during this time and then declined to the prestimulation value. The evoked tritium efflux was $2.94\pm0.27\%$ for S_1 and $2.79\pm0.23\%$ for S_2, not significantly different from each other; the calculated ratio S_2/S_1 was 0.95 ± 0.04. Under these conditions, the tritium outflow, induced by electrical stimulation, was significantly decreased by tetrodotoxin 1 μM ($S_2/S_1=0.05\pm0.03$, *P*<0.05) or ω-conotoxin 0.01 μM ($S_2/S_1=0.29\pm0.04$, *P*<0.05) as well as by exposure of ileal preparations to low Ca^{2+} concentrations in the superfusion solution (1.5 mM: $S_2/S_1=0.68\pm0.05$, *P*<0.05; 0.8 mM: $S_2/S_1=0.21\pm0.03$, *P*<0.05). Therefore, it was assumed that, following the application of electrical stimuli, radiolabelled acetylcholine undergoes an exocytotic release operated by extracellular Ca^{2+} influx into cholinergic axon terminals through voltage-dependent N-type Ca^{2+} channels. Neither the pharmacological agents nor lowering of Ca^{2+} concentration modified the resting overflow of tritium (not shown).

Table 1 Effects of histamine, histamine receptor agonists and histamine receptor antagonists on electrically evoked [³H]acetylcholine release. Longitudinal muscle strips of guinea pig ileum were preincubated with [³H]choline, superfused with Krebs solution and subjected twice (S_1 and S_2) to electrical field 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 ± SEM of 5–12 experiments

|	Electrically evoked tritium overflow (S_2/S_1)
Control	0.95 ± 0.04
Histamine (1 μM)	0.42 ± 0.03*
Pyridylethylamine (1 μM)	0.99 ± 0.05
Impromidine (1 μM)	0.97 ± 0.06
R-α-methylhistamine (1 μM)	0.48 ± 0.04*
Pyrilamine (1 μM)	0.96 ± 0.08
Cimetidine (10 μM)	1.04 ± 0.05
Thioperamide (1 μM)	0.99 ± 0.06
Clobenpropit (1 μM)	1.03 ± 0.06

*P<0.05: significant difference from control value

Effects of histamine, histamine receptor agonists and histamine receptor antagonists

Histamine, applied at the concentration of 1 μM, inhibited the evoked tritium outflow by 55.8% (Table 1). The inhibitory effect of histamine was mimicked by *R*-α-methylhistamine (H_3 receptor agonist), but not by pyridylethylamine (H_1 receptor agonist) or impromidine (H_2 receptor agonist). 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).

When tested at increasing concentrations (from 0.0001 μM to 100 μM), both histamine and *R*-α-methylhistamine yielded inhibitory concentration-response curves, with EC_{50} values of 33.5 nM and 41.6 nM, and E_{max} values of 59.7% and 54.8%, respectively. 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; Fig. 1). The following pK_d values were estimated for the antagonism of thioperamide and clobenpropit against histamine or *R*-α-methylhistamine: 8.31 (thioperamide vs. histamine); 8.53 (thioperamide vs. *R*-α-methylhistamine); 9.44 (clobenpropit vs. histamine); 9.32 (clobenpropit vs. *R*-α-methylhistamine). By contrast, pyrilamine (1 μM) or cimetidine (10 μM) did not modify the inhibitory effects of histamine or *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).

Fig. 1 Effects of increasing concentrations of **A** histamine or **B** R-α-methyl-histamine, 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]choline. Each point represents the mean of 4–6 experiments ± SEM (*vertical bars*)
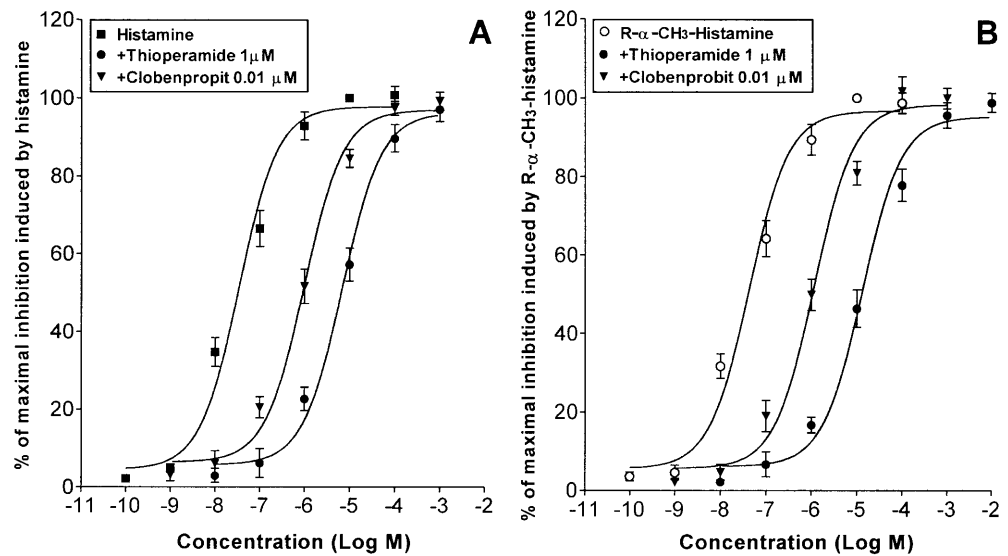
Effects of histamine on longitudinal muscle strips of ileum pretreated with pertussis toxin or N-ethylmaleimide

In control experiments, where ileal strips were incubated for 6 h in the absence of pertussis toxin, the electrically induced tritium efflux (S_1=3.14±0.31%; n=8) was not significantly different from that measured under standard conditions (S_1=2.94±0.27%; n=12). In addition, tritium outflow evoked by S_1 was not significantly affected by pretreatment of ileal preparations with pertussis toxin (3 μg/ml), N-ethylmaleimide (30 μM), dithiothreitol (100 μM)

or dithiothreitol plus N-ethylmaleimide (S_1=3.42±0.48%, 3.49±0.55%, 2.89±0.36% and 3.17±0.51%, 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, indicating that the viability of ileal tissues and their responsiveness to electrical stimulation were not affected by exposure to the above drugs.

The inhibitory effect of histamine (1 μM) on tritium overflow, elicited by electrical stimulation, was attenuated, but not abolished, by pretreatment with pertussis toxin (Fig. 2), and it was completely prevented by N-ethylmaleimide (Fig. 3). Dithiothreitol did not interfere with the inhibitory action of histamine on the evoked [^{3}H]acetyl-
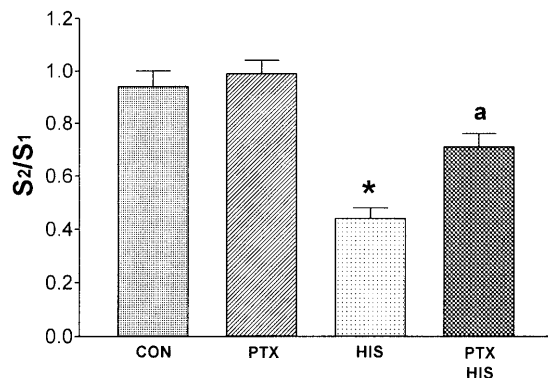

Fig. 2 Effect of pertussis toxin (*PTX*, 3 μg/ml) on the inhibitory action exerted by histamine (*HIS*, 1 μM) on the electrically evoked tritium outflow from longitudinal muscle strips of guinea pig ileum preincubated with [^{3}H]choline. Ileal preparations were exposed to pertussis toxin for 6 h and were then subjected to repeated washings for 1 h before incubation with [^{3}H]choline. Tissues used for control experiments or exposed to histamine alone were incubated for 6 h in the absence of pertussis toxin and underwent repeated washings for 1 h before loading with [^{3}H]choline. Histamine was added to the superfusion solution before S_2, from the 12th collection period onward. Each *column* represents the mean of 5–8 experiments ± SEM (*vertical bars*). *P<0.05: significant difference from control values (*CON*); ^{a}P<0.05: significant difference from histamine alone (*HIS*)
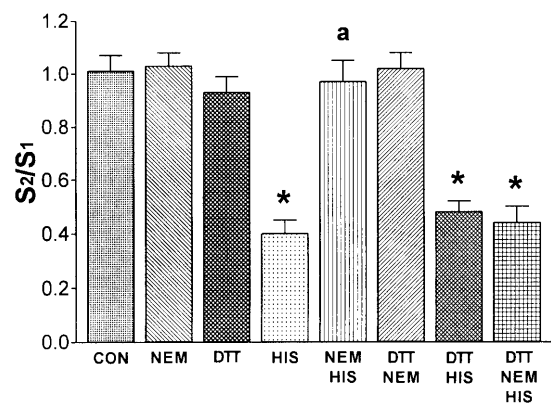

Fig. 3 Effects of N-ethylmaleimide (*NEM*, 30 μM), either alone or in the presence of dithiothreitol (*DTT*, 100 μM), on the inhibitory action exerted by histamine (*HIS*, 1 μM) on the electrically evoked tritium outflow from longitudinal muscle strips of guinea pig ileum preincubated with [^{3}H]choline. Ileal preparations were exposed to N-ethylmaleimide and dithiothreitol for 45 min and 50 min, respectively, before incubation with [^{3}H]choline. Histamine was added to the superfusion solution before S_2, from the 12th collection period onward. Each *column* represents the mean of 5–7 experiments ± SEM (*vertical bars*). *P<0.05: significant difference from control values (*CON*); ^{a}P<0.05: significant difference from histamine alone (*HIS*)

choline release, but totally counteracted the blocking action exerted by *N*-ethylmaleimide against the inhibitory effect of histamine (Fig. 3). 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 [³H]acetylcholine release. When tested alone, both tetraethylammonium (1 mM) and 4-aminopyridine (1 mM) significantly enhanced the evoked tritium outflow (+54.2% and +98.7%, respectively), these effects being counterbalanced by a reduction of Ca^{2+} concentration in the super-

fusion solution (from 2.5 mM to 1.5 mM or 0.8 mM, respectively; Fig. 4). Since tetraethylammonium and 4-aminopyridine exerted excitatory effects on [³H]acetylcholine release, the inhibitory action of histamine (1 μM) on the electrically induced tritium outflow was apparently counteracted by these K^+ channel blockers in experiments where the superfusion solution contained 2.5 mM Ca^{2+} (Fig. 4). However, in the presence of tetraethylammonium or 4-aminopyridine, the inhibitory effect of histamine was again evident when the ileal preparations were exposed to low Ca^{2+} concentration (1.5 mM or 0.8 mM, respectively) in order to counterbalance the increase in the evoked tritium efflux produced by these K^+ channel blockers (Fig. 4). The resting outflow was moderately increased by both tetraethylammonium and 4-aminopyridine (+9.2% and +17.8%, respectively).

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

When tested at the concentration of 0.001 μM, ω-conotoxin reduced the increase in tritium efflux elicited by electrical stimulation (–26.6% vs. control) and significantly enhanced the histamine-induced inhibition of the evoked tritium output (histamine vs. control: –52.1%; histamine plus ω-conotoxin vs. ω-conotoxin alone: –82.6%; Fig. 5). By contrast, nifedipine (0.01 μM) had no effect on the evoked tritium outflow and did not interfere with the inhibitory action of histamine (Fig. 5). Similar results were obtained when nifedipine was assayed at 0.1 μM or 1 μM (not shown). Additional experiments showed that the extent of the inhibitory action, exerted by histamine on the electrically induced [³H]acetylcholine release, increased as the Ca^{2+} concentration in the superfusing Krebs solution was lowered from the 9th collection period onward.
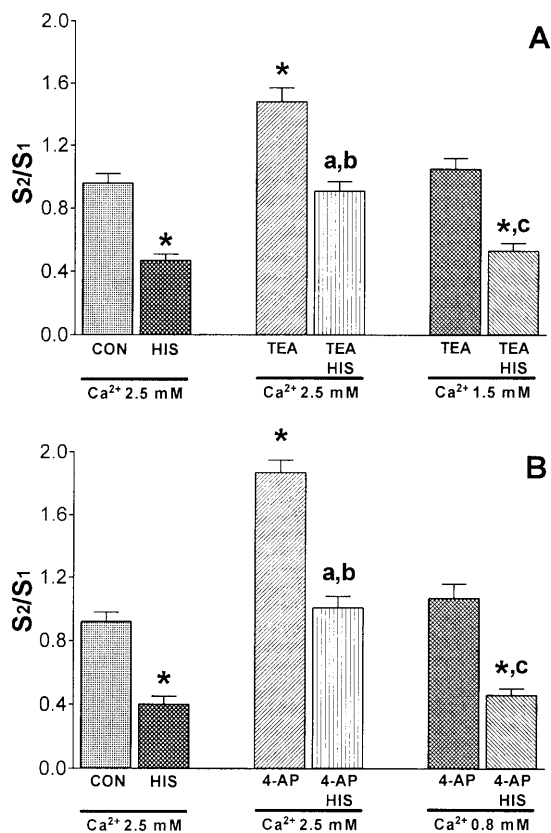

Fig. 4 Effects of **A** tetraethylammonium (*TEA*, 1 mM), tested in the presence of 2.5 mM or 1.5 mM Ca^{2+} in the superfusion solution, and **B** 4-aminopyridine (*4-AP*, 1 mM), tested in the presence of 2.5 mM or 0.8 mM Ca^{2+} in the superfusion solution, on the inhibitory action exerted by histamine (*HIS*, 1 μM) on the electrically evoked tritium outflow from guinea pig ileum longitudinal muscle strips preincubated with [³H]choline. Each *column* represents the mean of 5–8 experiments ± SEM (*vertical bars*). *$P<0.05$: significant difference from control values (*CON*); ᵃ$P<0.05$: significant difference from histamine alone (*HIS*); ᵇ$P<0.05$: significant difference from TEA or 4-AP in the presence of 2.5 mM Ca^{2+}; ᶜ$P<0.05$: significant difference from TEA or 4-AP in the presence of 1.5 mM or 0.8 mM Ca^{2+}, respectively
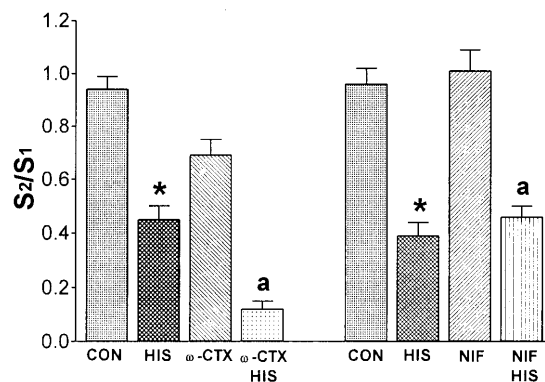

Fig. 5 Effects of ω-conotoxin (*ω-CTX*, 0.001 μM) or nifedipine (*NIF*, 0.01 μM) on the inhibitory action exerted by histamine (*HIS*, 1 μM) on the electrically evoked tritium outflow from longitudinal muscle strips of guinea pig ileum preincubated with [³H]choline. Each *column* represents the mean of 5–7 experiments ± SEM (*vertical bars*). *$P<0.05$: significant difference from control values (*CON*); ᵃ$P<0.05$: significant difference from ω-conotoxin or nifedipine alone
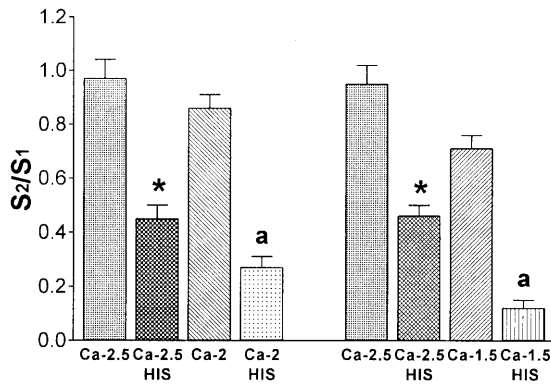
Fig. 6 Effects of the reduction of Ca^{2+} concentration in the superfusion solution from 2.5 mM to 2 mM (*Ca-2*) or 1.5 mM (*Ca-1.5*) on the inhibitory action exerted by histamine (*HIS*, 1 μM) on the electrically evoked tritium outflow from longitudinal muscle strips of guinea pig ileum preincubated with [³H]choline. Each *column* represents the mean of 5–9 experiments ± SEM (*vertical bars*). *$P<0.05$: significant difference from control values obtained with Ca^{2+} at 2.5 mM (*Ca-2.5*); $^aP<0.05$: significant difference from control values obtained with Ca^{2+} at 2 mM (*Ca-2*) or 1.5 mM (*Ca-1.5*)

In particular, the degree of histamine-induced inhibition of [³H]acetylcholine release accounted for –68.6% and –83.1% when the ileal preparations were exposed to 2 mM and 1.5 mM Ca^{2+}, respectively (Fig. 6). ω-Conotoxin, nifedipine and reduction of Ca^{2+} concentration in the superfusion solution did not significantly alter the resting tritium outflow (not shown).

Effects of histamine in the presence of drugs acting on adenylyl cyclase, serine/threonine protein kinase and tyrosine kinase pathways

Tritium efflux induced by S_1 was not modified by exposure of ileal strips to rolipram (100 μM), PMA (1 μM), H-89 (10 μM), Ro-31-8220 (1 μM), KT5823 (1 μM) or lavendustin A (10 μM), whereas it was increased by 52.7% and 46.2% in the presence of forskolin (10 μM) and 8-Br-cAMP (1 mM), respectively (Table 2). In all cases S_2/S_1 values were not significantly different from those obtained in control experiments (Table 2). No relevant changes in the resting tritium output were detected in the presence of PMA, Ro-31-8220, KT5823 or lavendustin A (not shown). However, forskolin, 8-Br-cAMP or rolipram moderately enhanced (+21.6%, +16.3% and +11.4%, respectively) and H-89 reduced (–13.8%) the spontaneous tritium outflow. Under these conditions, none of the above drugs were able to significantly interfere with the inhibitory action exerted by histamine (1 μM) on the electrically evoked release of [³H]acetylcholine (Table 2).

Discussion

The present study provides evidence that histamine H_3 receptors, involved in the regulation of intestinal choliner-

Table 2 Effects of drugs active on cyclic AMP, serine/threonine protein kinase or tyrosine kinase pathways on the histamine-induced inhibition of electrically evoked [³H]acetylcholine release. Longitudinal muscle strips of guinea pig ileum were preincubated with [³H]choline, superfused with Krebs solution and subjected twice (S_1 and S_2) to electrical field stimulation. The effects of test drugs on tritium outflow, evoked by electrical 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 ± SEM of 5–7 experiments

	Electrically evoked tritium overflow		
	S_1	S_2/S_1	
		Histamine (μM)	
		0	1
Control	2.79±0.34	0.97±0.05	0.45±0.03**
Forskolin (10 μM)	4.26±0.49*	0.93±0.06	0.41±0.05**
8-Br-cAMP (1 mM)	4.08±0.38*	0.95±0.08	0.43±0.04**
Rolipram (100 μM)	2.93±0.22	1.04±0.07	0.46±0.05**
PMA (1 μM)	3.12±0.38	0.98±0.05	0.42±0.04**
H-89 (10 μM)	2.64±0.33	0.94±0.06	0.47±0.06**
Ro-31-8220 (1 μM)	2.88±0.45	0.96±0.06	0.42±0.06**
KT5823 (1 μM)	3.11±0.41	1.03±0.05	0.48±0.05**
Lavendustin A (10 μM)	3.05±0.28	1.01±0.04	0.39±0.07**

*$P<0.05$: significant difference from control value
**$P<0.05$: significant difference from S_2/S_1 values obtained in the absence of histamine

gic neurotransmission, are coupled to G_i/G_o proteins. Therefore, taking into account the mechanisms currently proposed for the regulation of neurotransmitter release by G proteins (Miller 1998), efforts were made to elucidate the possible role played by K^+ or Ca^{2+} channels as well as by various signal transduction pathways in the modulation of acetylcholine release by H_3 receptors.

The pharmacological profile of histamine receptors characterized in the present study conforms to the criteria for classification of H_3 receptors (Hill et al. 1997): (1) the inhibitory effect of histamine on tritium outflow was mimicked by R-α-methylhistamine, but not by pyridylethylamine or impromidine; (2) the inhibitory actions of both histamine and R-α-methylhistamine were antagonized by thioperamide or clobenpropit, whereas they were insensitive to antagonists for H_1 or H_2 receptors. These findings agree with previous studies dealing with presynaptic modulation of cholinergic neurotransmission by H_3 receptors (Fuder and Muscholl 1995; Hill et al. 1997). For instance, the potencies calculated for thioperamide and clobenpropit are comparable with those found by other authors in functional assays on isolated intestinal preparations (Leurs et al. 1991; Schlicker et al. 1994a). In addition, our estimated potencies for thioperamide and clobenpropit are consistent with values obtained for the same drugs in [³H]acetylcholine or [³H]noradrenaline release assays from central nervous system tissues (Clapham and Kilpatrick 1992; Alves-Rodriguez et al. 1998).

It is remarkable that, in the present study, very similar EC_{50} values were obtained for histamine (33.5 nM) and R-α-methylhistamine (41.6 nM), whereas in other reports R-α-methylhistamine was more potent than histamine (for review, see Leurs et al. 1995; Hill et al. 1997). At present, we do not have a clear explanation for these differences. However, in previous studies performed on guinea pig intestine, EC_{50} values of approximately 40–770 nM and 5–25 nM have been estimated for histamine and R-α-methylhistamine, respectively (Trzeciakowski 1987; Menkveld and Timmerman 1990; Poli et al. 1991). On this basis, although the EC_{50} values obtained in the present study do not fall exactly within the above ranges, it is conceivable that the discrepancies between our data and those reported by other authors might be due to experimental variability.

Binding experiments 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 (West et al. 1990). Subsequent studies confirmed this subclassification and showed that other antagonists, including clobenpropit, are not able to differentiate between H_{3A} and H_{3B} receptor subtypes (Schlicker et al. 1994a). These studies also demonstrated that noradrenaline release in mouse brain cortex is modulated by H_{3A} receptor subtypes. In spite of these findings, the heterogeneity of H_3 receptors is still a matter of debate (Hill et al. 1997) and no conclusive evidence of functional H_{3B} receptors has been obtained. In the present study, the potencies of thioperamide and clobenpropit against histamine or R-α-methylhistamine would suggest that intestinal cholinergic neurotransmission is modulated by H_3 receptors resembling the H_{3A} subtype.

In the present series of experiments, neither thioperamide nor clobenpropit affected the resting or electrically induced tritium outflow, indicating that endogenous histamine does not exert a tonic inhibitory effect on intestinal cholinergic neurotransmission. Conflicting data regarding an effect of endogenous histamine on cholinergic transmission have been previously reported. In agreement with our findings, Schlicker et al. (1994a) showed that thioperamide and clobenpropit did not alter the cholinergic twitch responses of guinea pig ileum. However, thioperamide enhanced the evoked [³H]acetylcholine release from guinea pig ileum (Poli et al. 1991) or rat brain cortex (Clapham and Kilpatrick 1992). The discrepancy between our results and those reported by Poli et al. (1991) may be due to the different experimental conditions used in these studies. Indeed, the electrical stimulation applied to ileal preparations during incubation with [³H]choline in our studies might deplete endogenous histamine stores, thus causing an underestimation of the enhancing action of thioperamide on [³H]acetylcholine release.

Previous binding studies provided 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 [³H]acetylcholine release was attenuated by pertussis toxin and abolished by N-ethylmaleimide, suggest that the H_3 receptors, located on cholinergic nerve endings of guinea pig ileum, are coupled to G proteins belonging to G_i/G_o subtypes. This finding is consistent with the results of previous studies where the involvement of G_i/G_o proteins was demonstrated for the H_3 receptor-mediated modulation of noradrenergic neurotransmission (Nozaki and Sperelakis 1989; Endou et al. 1994; Schlicker et al. 1994b; Blandizzi et al. 2000).

In the case of presynaptic receptors linked to G proteins, three major mechanisms have been proposed for the modulation of neurotransmitter release: (1) activation of K^+ channels, resulting in a reduction of action potential efficacy; (2) inhibition of Ca^{2+} channels, leading to an impairment of exocytotic machinery; (3) direct modulation of the vesicle release apparatus (Miller 1998).

As far as K^+ channels are concerned, in the present study care was taken to assay the effects of tetraethylammonium and 4-aminopyridine on the evoked tritium outflow, since it is known that the blockade of voltage-dependent K^+ channels prolongs the duration of action potential, thus causing an increment of Ca^{2+} entry into axon terminals, with a consequent enhancement of neurotransmitter release (Starke et al. 1989). As expected, both tetraethylammonium and 4-aminopyridine enhanced the electrically induced [³H]acetylcholine release, and this effect could be counterbalanced by an appropriate reduction of Ca^{2+} concentration in the superfusion solution, confirming that the potentiating actions of these K^+ channel blockers are indirect in nature and depend on an increased Ca^{2+} influx. These findings allowed a correct interpretation of data obtained from experiments where the inhibitory effect of histamine on [³H]acetylcholine release was examined in the presence of K^+ channel blockade. Indeed, under these conditions, tetraethylammonium or 4-aminopyridine caused an apparent attenuation of the histamine-induced inhibitory effect, which was ascribed to an increment of intraneuronal Ca^{2+} concentration induced by the prolongation of action potential. A consistent support to this view came from experiments in which the increment of tritium outflow, produced by tetraethylammonium or 4-aminopyridine, was counterbalanced by a decrease in Ca^{2+} concentration in the superfusion solution and, as a consequence, the modulatory action of histamine on [³H]acetylcholine release was again well evident. On this basis, since neither tetraethylammonium nor 4-aminopyridine inhibited the modulatory effect of histamine, a coupling of H_3 receptors to voltage-dependent K^+ channels appears to be unlikely.

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 cholinergic nerve endings, may account for the H_3 receptor-mediated inhibition of evoked [³H]acetylcholine release from ileal preparations: (1) the inhibitory effect of histamine was markedly enhanced by ω-conotoxin, a selective blocker of N-type Ca^{2+} channels, which play a pivotal role in the regulation of exocytotic neurotransmitter release (Dolphin 1995), whereas the blockade of L-type Ca^{2+} channels by nifedipine was without consequences; (2) lowering of Ca^{2+} concentration in the superfusion so-

lution significantly enhanced the inhibitory action of histamine. These findings, taken together with data reported from previous studies on duodenal motility (Poli et al. 1994), indicate that presynaptic H_3 receptors inhibit N-type Ca^{2+} channels, causing a reduced availability of extracellular Ca^{2+} ions for stimulus-release coupling at the level of intestinal cholinergic terminals.

In general, receptors coupled to G_i/G_o proteins 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 cell lines transfected with human or rat H_3 receptor cDNA (Lovenberg et al. 1999, 2000), but other authors failed to observe an inhibition of adenylyl cyclase activity in studies on native H_3 receptors (Garbarg et al. 1989; Marley et al. 1991; Schlicker et al. 1994b; Celuch 1995). Cherifi et al. (1992) described a negative coupling of H_3 receptors to phospholipase C in human HGT-1 cells, but no evidence of protein kinase C involvement was obtained for H_3 receptors modulating glutamate release at a central level (Brown and Haas 1999). More recently, the molecular cloning of guinea pig H_3 receptor has led to the identification of two receptor isoforms, differing by a 30-amino-acid stretch within the third intracellular loop, the molecular domain that in G protein-dependent receptors is thought to be responsible for coupling to the signal transduction pathway (Tardivel-Lacombe et al. 2000).

The above findings prompted us to assay various pharmacological tools to explore the possibility that the activation of presynaptic H_3 receptors interferes with the activity of adenylyl cyclase (forskolin, 8-Br-cAMP, rolipram), serine-threonine protein kinases (PMA, H-89, Ro-31-8220, KT5823) or tyrosine kinase (lavendustin A). However, none of these drugs was able to modify the inhibitory effect of histamine on the evoked [³H]acetylcholine release. Therefore, since the inhibition of Ca^{2+} channels by G_i/G_o proteins may involve a direct action of the βγ-subunits of the G protein on the β-subunit of voltage-dependent Ca^{2+} channels (Dolphin 1998), it is conceivable that in enteric cholinergic neurons G_i/G_o proteins, coupled to H_3 receptors, inhibit Ca^{2+} influx into nerve endings through a direct interaction with N-type Ca^{2+} channels. On these bases, the present study, as well as the results of previous reports (Marley et al. 1991; Cherifi et al. 1992; Schlicker et al. 1994b), argue against the possibility that H_3 receptors are coupled to adenylyl cyclase or phospholipase C pathways at the level of central or peripheral sites. It remains then to be established whether the results obtained with cloned H_3 receptors (Lovenberg et al. 1999, 2000) might be ascribed to transfection artifacts or rather reflect the existence of alternative transduction mechanisms for native H_3 receptors in different species and/or anatomical locations.

In conclusion, the present study identified the mechanisms through which the activation of presynaptic H_3 receptors, located on cholinergic nerve endings, caused an inhibitory modulation of intestinal cholinergic neurotransmission. 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 the level of ileal cholinergic nerve terminals.

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