0026-895X/02/6101-239–245$3.00
MOLECULAR PHARMACOLOGY
Vol. 61, No. 1
1245/958070
Mol Pharmacol 61:239–245, 2002
Printed in U.S.A.

Presynaptic H_3 Autoreceptors Modulate Histamine Synthesis through cAMP Pathway

JORDI GOMEZ-RAMIREZ, JORDI ORTIZ, and ISAAC BLANCO

Department of Biochemistry and Molecular Biology, School of Medicine, Universitat Autònoma de Barcelona, Bellaterra, Spain.

Received July 30, 2001; accepted October 19, 2001.

This paper is available online at http://molpharm.aspetjournals.org

ABSTRACT

Histamine H_3 receptors modulate histamine synthesis, although little is known about the transduction mechanisms involved. To investigate this issue, we have used a preparation of rat brain cortical miniprisms in which histamine synthesis can be modulated by depolarization and by H_3 receptor ligands. When the miniprisms were incubated in presence of forskolin, dibutyryl-cAMP, or 3-isobutyl-1-methylxanthine (IBMX), histamine synthesis was stimulated in 34, 29, and 47%, respectively. These stimulations could be prevented by the selective cAMP protein kinase blocker *R*p-adenosine 3′,5′-cyclic monophosphothioate triethylamine (*R*p-cAMPs). Preincubation with the H_3 receptor agonist imetit prevented IBMX- (100% blockade) and forskolin- (70% blockade) induced stimulation of histamine synthesis. The H_3 inverse agonist thioperamide enhanced histamine synthesis in the presence of 1 mM IBMX or 30 mM potassium (+47 and +45%, respectively). Similarly, the H_3 antagonist clobenpropit enhanced histamine synthesis in the presence of 30 mM potassium (+ 59%). The cAMP-dependent protein kinase blockers *R*p-cAMPs and PKI14–22 could impair the effects of thioperamide and clobenpropit, respectively. These results indicate that the adenylate cyclase-protein kinase A pathway is involved in the modulation of histamine synthesis by H_3 autoreceptors present in histaminergic nerve terminals.

Histamine (HA) is one of the aminergic neurotransmitters playing an important role in the regulation of several physiological and pathological processes. In the mammalian brain, histamine is synthesized by a population of neurons whose cell bodies are restricted to the tuberomamillary nuclei of the posterior hypothalamus (Schwartz et al., 1991). These histaminergic neurons have been implicated in several brain functions (e.g., sleep/wakefulness, hormonal secretion, cardiovascular control, thermoregulation, food intake, and memory formation). In peripheral tissues, histamine is stored in mast cells, basophils, and enterochromaffin-like cells. In these tissues, histamine is considered one of the most important mediators of allergy and inflammatory responses acting through H_1 receptors (Black et al., 1972) as well as in gastric acid secretion acting through H_2 receptors (Ash and Schild, 1966).

Histamine H_3 receptors were first identified as presynaptic autoreceptors on brain histamine neurons, although they are also present in many other cell types (Schwartz et al., 1991). H_3 autoreceptors control the stimulated release of histamine (Arrang et al., 1983) as well as histamine synthesis (Arrang et al., 1987b). Histamine formation from its precursor histidine is catalyzed by the enzyme L-histidine decarboxylase (HDC; EC 4.1.1.22). The cDNA of rat HDC (Joseph et al., 1990) encodes a protein with a molecular mass of 74 kDa (655 amino acid residues) that is post-translationally processed to obtain an active enzyme of 54 kDa (469 amino acid residues). The HDC sequence has two regions fitting consensus phosphorylation sites for PKA. This suggests the possibility that PKA may participate in HDC modulation, although very little is known about the mechanisms involved in the regulation of the enzyme. The cloning and functional characterization of human and rat H_3 receptor cDNA (Lovenberg et al., 1999, 2000) show that the receptor belongs to the family of G-protein-coupled receptors. In these studies, H_3 receptor agonists decreased cAMP accumulation elicited by forskolin stimulation of adenylate cyclase in receptor-transfected cells. Also, it had been observed previously that stimulation of [^{35}S]GTPγ[S] binding through H_3 receptor agonists in cerebral cortical membranes was prevented by pertussis toxin (Clark and Hill, 1996). All these results demonstrate that histamine H_3 receptors coupled negatively to adenylate cyclase through $G_{i/o}$ proteins. Nevertheless, is not known whether H_3 autoreceptors in histaminergic neurons regulate histamine synthesis through cAMP formation. If this hypoth-

This work was supported by grants FIS95–1737 from Fondo de Investigaciones Sanitarias and 98/119–00 from Fundación La Caixa.

ABBREVIATIONS: HA, histamine; HDC, histidine decarboxylase; PKA, cAMP-dependent protein kinase; AC, adenylate cyclase; db-cAMP, dibutyryl-cAMP; IBMX, 3-isobutyl-1-methylxanthine; *R*p-cAMPS, *R*p-adenosine 3′,5′-cyclic monophosphothioate triethylamine; HPLC, high performance liquid chromatography; PKI_{14-22}, myristoylated cAMP-dependent protein kinase inhibitor 14–22 amide; KRM, Krebs-Ringer-bicarbonate medium.

esis is true, high cAMP levels should stimulate histamine synthesis. However, some evidence against that was presented by Huszti et al. (1991), who observed that HDC activity could be decreased in phosphorylating conditions [including ATP, Mg_2Cl, cAMP, and cAMP-dependent protein kinase (PKA)].

To investigate the role of adenylate cyclase (AC)-PKA pathway in H_3 receptor-regulated histamine synthesis, we prepared rat brain miniprisms as described originally by Arrang et al. (1983). In our preparations, we confirmed that [^{3}H]histamine synthesis is stimulated by depolarization and modulated by H_3 receptor ligands as reported previously by Arrang et al. (1987a). Subsequently, we studied the effects of stimulators and inhibitors of AC-PKA pathway, such as forskolin, dibutyryl-cAMP (db-cAMP), 3-isobutyl-1-methylxanthine (IBMX), or *R*p-adenosine 3′,5′-cyclic monophosphothioate triethylamine (*R*p-cAMPS) on histamine synthesis in nondepolarizing conditions. Finally, we tested the effect of H_3 receptor ligands on cAMP-stimulated histamine synthesis.

Experimental Procedures

Materials. Ring-labeled [2,5-^{3}H]L-histidine stocks (1 mCi; 50 Ci/mmol) were obtained from Amersham Biosciences (Little Chalfont, Buckinghamshire, UK) and were purified by high performance liquid chromatography (HPLC) before their use (Ortiz et al., 2000). Nonradiolabeled histidine, histamine, thioperamide maleate, imetit, clobenpropit dihydrobromide, *R*p-cAMPs triethylamine salt, octanesulfonic acid, and trichloroacetic acid were purchased from Sigma/RBI (Steinheim, Germany). $PKI_{14–22}$, db-cAMP, and IBMX were obtained from Calbiochem/Merck KGaA (Darmstadt, Germany). Forskolin was supplied from Alomone-Labs (Jerusalem, Israel). IBMX and forskolin stocks were prepared in dimethyl sulfoxide. All other reagents were of the maximum purity available. A reversed-phase C18 HPLC column, 25 × 0.46 cm (Tracer Extrasil ODS-2, 5-μm particle size) equipped with a 2 × 20 mm guard column (Upchurch) was purchased from Teknokroma (Barcelona, Spain). Amberlite IRA-900 (mesh 16–50) strong anion exchange resin (Supelco) was also from Teknokroma. Microspin filter Ultrafree-MC tubes with low-binding Durapore membrane of 0.45-μm pore size were obtained from Millipore GmbH (Eschborn, Germany). OptiPhase "HiSafe"-3 liquid scintillation cocktail was purchased from PerkinElmer Wallac (Turku, Finland).

Preparation and Incubation of Brain Slices. Male Sprague-Dawley rats of 200 to 250 g (Servei d'Estabulari, Universitat Autonoma de Barcelona, Spain) were killed by decapitation between 9 and 10 AM. Brains were immediately placed into ice-cold modified Krebs-Ringer-bicarbonate medium (KRM) of the composition 120 mM NaCl, 0.8 mM KCl, 2.6 mM $CaCl_2$, 0.67 mM $MgSO_4$, 1.2 mM KH_2PO_4, 27.5 mM $NaHCO_3$, and 10 mM glucose, pH 7.4. Working in a cold environment (4°C), meninges were removed and cortical lobes were dissected without white matter. A McIlwain tissue chopper (Mickle Laboratory Engineering Co, Gomshall, Surrey, UK) was used to obtain cerebral cortex miniprisms of 0.3 × 0.3 mm. The miniprisms were suspended in KRM and washed three times with ice-cold KRM to remove debris of damaged cells. Then, the miniprisms were allowed to settle and the excess KRM was removed. Aliquots of 100 μl from the settled slice suspension (usually containing 2–3 mg of protein) were distributed (using a yellow pipette tip cut at 1 cm from the tip) into 2-ml polypropylene tubes. The tubes were preincubated for 25 min at 37°C in a shaking water bath under 95% O_2/5% CO_2 atmosphere. After preincubation, prepurified [^{3}H]histidine (6.25 μCi; final concentration, 0.5 μM) was added to all samples and the tubes were incubated for 5 min further to allow for [^{3}H]histidine uptake. Then, miniprisms were incubated for 30 min to synthesize [^{3}H]histamine in a final volume of 250 μl. If depolarizing conditions were required, the buffer added to bring volume to 250 μl contained concentrated KCl to make a final concentration of 30 or 60 mM potassium (K^+) and NaCl concentration was decreased proportionally to maintain the isotonicity.

When drugs were tested (as forskolin, db-cAMP, IBMX, *R*p-cAMPS, $PKI_{14–22}$, and H_3 receptor ligands) they were added 15 min before the incubation period. Several concentrations of each compound were tested, because the diffusion of any one of them might be very different across the relatively dense tissue slice preparations, which may explain why apparently high drug concentrations are needed to obtain intracellular effects. Incubations were stopped by placing samples on ice and adding 35 μl of deproteinization mixture (containing 25 μl of 10% trichloroacetic acid mixed with 10 μl of 10 mM histamine as internal standard). Then, they were sonicated for 10 to 20 s at 4°C using a Sonic Dismembrator (Dynatech Labs, Chantilly, VA). Blank sample incubations were stopped with deproteinization mixture before the addition of labeled histidine, and they were stored at 4°C during the incubation of the rest of samples. Finally, the samples were centrifuged at 12,000*g* for 10 min at 4°C and supernatants were recovered and processed as described below under *Histamine Purification by HPLC*.

Protein Quantification. To take into account the variability of miniprism amounts pipetted into each tube, protein was determined in an aliquot of each homogenate after sample sonication. Protein content was measured by the method of Lowry et al. (1951), using bovine serum albumin as standard.

Purification of [^{3}H]Histidine. Aliquots of the commercial [^{3}H]histidine standard were purified before their use to check specific activity and avoid excessive radiolysis (Ortiz et al., 2000). Briefly, the HPLC system (Kontron 325 pump) was set to perform a linear gradient from 1 to 6 mM sodium phosphate buffer (pH 3) in 12.5 min at a flow rate of 1 ml/min. A constant percentage of methanol (2%) and 0.1 mM octanesulfonic acid were present throughout. Under these conditions, histidine was eluted at 9 to 10 min and it was detected at 225 nm on a UV 432 detector (Kontron Instruments, Watford, Herts, UK). To calculate the quantity of the purified [^{3}H]histidine, a linear regression of nonradiolabeled histidine (0.2- to 20-nmol standards) versus their corresponding areas was performed. Finally, the specific activity of purified [^{3}H]histidine was determined by dividing total dpm obtained in the histidine fraction by the amount of histidine detected.

Histamine Purification by HPLC. [^{3}H]histamine levels in each sample were determined by HPLC separation after elimination of excess [^{3}H]histidine by ion-exchange on Amberlite IRA 900 resin (Ortiz et al., 2000). Briefly, 100 μl of resin, previously equilibrated with NaOH, was added to deproteinized supernatants into the top half of Ultrafree Microspin tubes. The tubes were vortexed for 10 min in a multitube shaker at room temperature. During this step, the resin bound approximately 85% of the [^{3}H]histidine. Finally, the tubes were centrifuged at 4,000*g* for 5 min and the filtrates recovered in the bottom half of tubes were injected into the HPLC system.

The HPLC procedure was as performed as described previously (Ortiz et al., 2000). In short, the HPLC system was equipped with a reverse-phase C18 column (Tracer Extrasil ODS2; 5-μm particle size; 25 × 0.46 cm) and a 2 × 20 mm guard column. The ion-pair mobile phase was made up of 21% (v/v) methanol, 10 mM octanesulfonic acid, and 0.3 M sodium phosphate buffer, adjusted to pH 3 with phosphoric acid. The flow rate during sample injection was 1 ml/min. Under these conditions, histamine eluted at 10 to 11 min and it was detected by the internal standard UV absorbance at 225 nm. All the collected fractions were mixed with Optiphase scintillation cocktail and dpm were counted. For each sample, the internal standard histamine peak area was compared with the external standard histamine peak area to obtain the recovery during sample processing (typically about 75%). To quantify the [^{3}H]histamine synthesis in each sample, dpm obtained in the fraction collected were corrected by recovery of the histamine internal standard, blank dpm values, specific activity of the labeled histidine, protein content, and sample

incubation time. Most drugs used in this work did not interfere in the histamine purification procedure except IBMX (1 mM), which had a retention time of ~50 min, so it had to be taken into account when successive samples were injected.

Statistical Analysis. Concentration-response curves obtained with H_3 receptor ligands (imetit or thioperamide) in depolarizing conditions were analyzed by nonlinear regression using Prism (GraphPad Software, San Diego, CA). Each concentration-response curve was fitted to a logistic function of the form: $E = E_{max}[A]^{n_H} / ([A]^{n_H} + (EC_{50})^{n_H})$, where [A] is the ligand concentration, E is the agonist effect, E_{max} is the maximal response, n_H is the Hill coefficient, and EC_{50} is the ligand concentration giving half-maximal response.

Statistical significance of differences between values was evaluated by analysis of variance followed by Dunnett's test for multiple group comparisons. Significance was established at $P < 0.05$.

Results

Potassium Depolarization Stimulates Histamine Synthesis. To confirm data reported previously (Arrang et al., 1987b), we studied the effect of depolarization with potassium on histamine synthesis in rat brain cortical miniprisms. Potassium produced a dose-dependent increase of histamine synthesis, reaching the maximal effect at 60 mM K^+ (+192 ± 19.5% versus 2 mM K^+). Histamine synthesis in samples with 2, 30, and 60 mM K^+ was 20 ± 0.5, 41 ± 0.9 and 59 ± 1.8 fmol of HA/mg of protein/h, respectively. All groups were statistically different from 2 mM K^+ ($P < 0.01$).

Imetit and Thioperamide Modulate Potassium-Stimulated Histamine Synthesis. To assess that presynaptic H_3 receptors were pharmacologically functional in our preparations, we studied the effects of an agonist or an inverse agonist on histamine synthesis in potassium-stimulated conditions (30 mM K^+). Imetit, a potent and selective H_3 receptor agonist (Garbarg et al., 1992), inhibited the K^+-stimulated histamine synthesis in a dose-dependent manner. The maximal inhibition (75%) was obtained at of 100 nM and the half-maximal inhibitory concentration was 5.8 nM (Fig. 1A). Conversely, the H_3 antagonist thioperamide (Arrang et al., 1987a), recently classified as H_3 inverse agonist (Morisset et al., 2000), increased the K^+-stimulated histamine synthesis in a dose-dependent way. Thioperamide maximal stimulation (50%) was reached at 10 nM and the half-maximal stimulatory concentration was 0.92 nM (Fig. 1B). Similar values were obtained in at least two or three additional experiments (not shown).

Forskolin, db-cAMP, and IBMX Stimulate Histamine Synthesis. Because HDC has two PKA consensus phosphorylation sites (Joseph et al., 1990), we studied the effect of several drugs increasing cAMP levels on [^{3}H]histamine synthesis. Forskolin (100 μM), a potent activator of AC that synthesizes cAMP from ATP (Daly et al., 1982; Seamon et al., 1983), elicited an increase of 34 ± 4.3% in the [^{3}H]histamine synthesis in nondepolarized conditions (2 mM K^+) (Fig. 2). db-cAMP (1 mM), a cell-permeable cAMP analog that directly activates PKA (Posternak and Weiman, 1974), also produced an increase of 29 ± 6.2% in the [^{3}H]histamine synthesis (Fig. 2). Finally, IBMX (1 mM), a nonspecific inhibitor of cAMP and cGMP phosphodiesterases (Beavo and Reifsnyder, 1990; Morgan et al., 1993), elicited an [^{3}H]histamine increase of 47 ± 5.6% (Fig. 2). In addition, we studied the effect of forskolin (100 μM) or db-cAMP (1 mM) on histamine synthesis in the presence of IBMX (1 mM). IBMX potentiated both forskolin- and db-cAMP-stimulation, producing increases of 69.6 ± 1.9% and 89.6 ± 8.1%, respectively.

***R*p-cAMPS Prevents Histamine Synthesis Stimulated by Forskolin and db-cAMP plus IBMX.** To confirm that cAMP-stimulated [^{3}H]histamine synthesis was mediated through PKA activation, we examined whether the PKA inhibitor (*R*p-cAMPS) could prevent the stimulatory effects produced by forskolin (100 μM) or db-cAMP (1 mM) plus IBMX (1 mM). *R*p-cAMPS (Botelho et al., 1988; Wang et al., 1991) decreased forskolin-stimulated [^{3}H]histamine synthesis in a dose-dependent manner, reaching its maximal inhibition (45%) at a concentration of 500 μM (Fig. 3). Similarly, the stimulatory effect produced by db-cAMP plus IBMX was

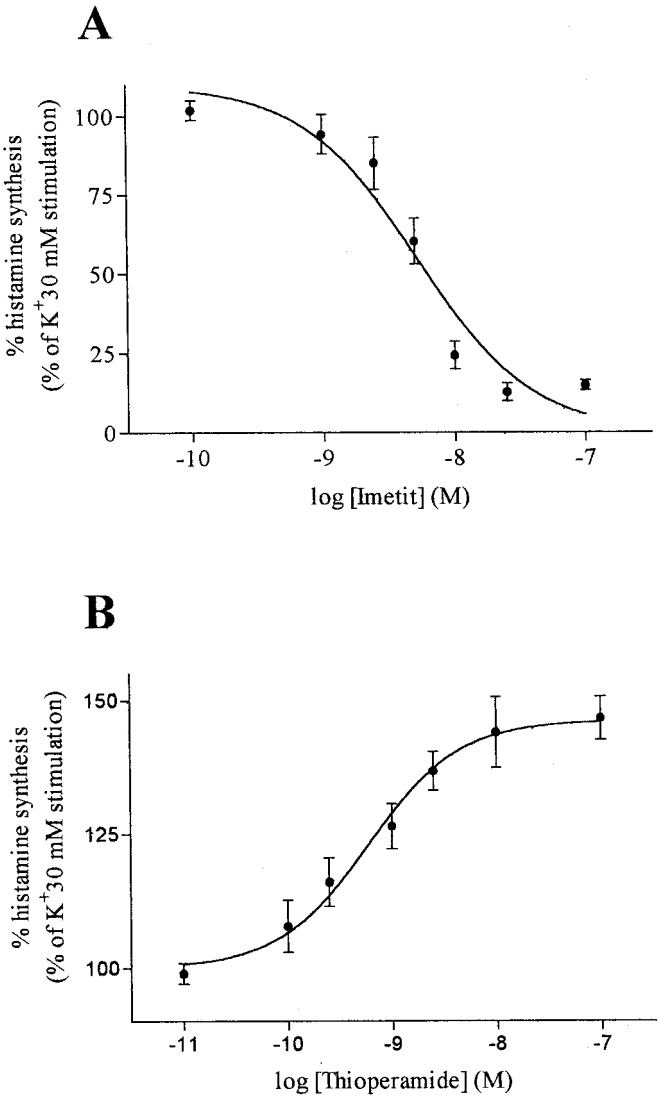

Fig. 1. Effects of H_3 receptor ligands on K^+-stimulated histamine synthesis. A, effect of imetit (agonist) on [^{3}H]histamine synthesis in 30 mM K^+ depolarized cerebral cortex miniprisms. B, effect of thioperamide (inverse agonist) on [^{3}H]histamine synthesis in depolarizing conditions. Results are means ± S.E.M. of two to four replicates. In both figures, 100% [^{3}H]histamine synthesis corresponds to the difference between 30 and 2 mM K+ and results are expressed as a percentage of this value. [^{3}H]histamine synthesis was 24 ± 0.2 and 48 ± 0.3 fmol of HA/mg of protein/h in the presence of 2 and 30 mM K^+, respectively. Similar results were obtained in at least two or three additional experiments (not shown).

also blocked dose dependently by the PKA inhibitor. In this case, *R*p-cAMPS (500 μM) blocked (80%) the effects of cotreatment with db-cAMP plus IBMX. *R*p-cAMPS alone did not statistically modify histamine synthesis in basal conditions.

Imetit Prevents Forskolin- and IBMX-Stimulated Histamine Synthesis. Because activation of recombinant H_3 receptors decreases intracellular cAMP levels (Lovenberg et al., 1999), we studied whether imetit (H_3 agonist) could prevent forskolin- or IBMX-stimulated [^{3}H]histamine synthesis. Imetit strongly blocked forskolin-stimulated synthesis in a dose-dependent manner. In the presence of 100 nM imetit, forskolin did not produce a statistically significant increase of [^{3}H]histamine synthesis compared with controls without forskolin (Fig. 4). Similarly, the IBMX-stimulation was also dose dependently prevented by imetit, which at 100 nM absolutely blocked IBMX effect. Imetit alone (at concentrations studied) did not statistically modify histamine synthesis under basal conditions (2 mM K^+).

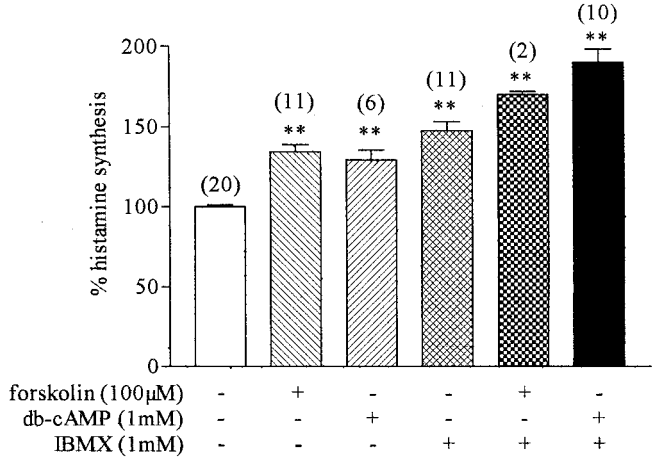

Fig. 2. Stimulators of AC-PKA pathway increase histamine synthesis. Forskolin, db-cAMP or IBMX were added to the medium 15 min previously to the incubation period. Results are means ± S.E.M. Number of replicates is indicated in brackets above the columns. **, $P < 0.01$ compared with controls (2 mM K^+). 100% [^{3}H]histamine synthesis corresponds to 20 ± 0.5 fmol of HA/mg of protein/h.

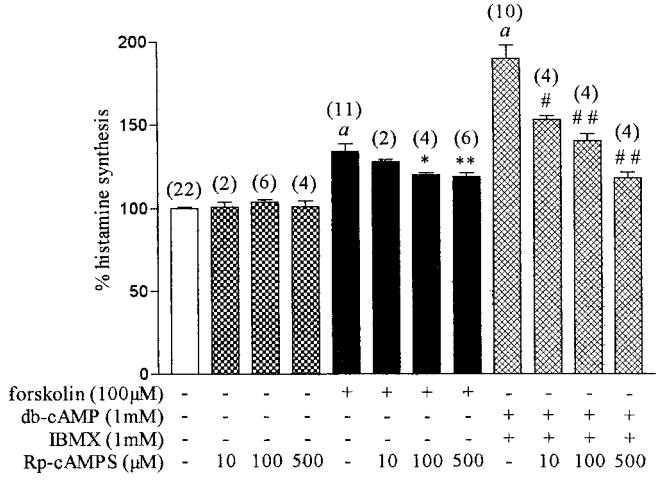

Fig. 3. The PKA inhibitor *R*p-cAMPS prevents forskolin- or db-cAMP plus IBMX-stimulated histamine synthesis. *R*p-cAMPS was added to the medium 5 min before the stimulatory drugs. Results represent means ± S.E.M. Number of replicates is indicated in brackets above the columns. *a*, $P < 0.01$ compared with controls (2 mM K^+). *, $P < 0.05$; **, $P < 0.01$ compared with forskolin stimulation. #, $P < 0.05$; ##, $P < 0.01$ compared with db-cAMP plus IBMX cotreatment. 100% [^{3}H]histamine synthesis in 2 mM K^+ was 20 ± 0.5 fmol of HA/mg of protein/h.

Thioperamide Increases IBMX- and K^+-Stimulated Histamine Synthesis. Thioperamide alone (H_3 inverse agonist) did not increase histamine synthesis in nondepolarizing conditions (2 mM K^+). Thus, we studied whether this drug could show effects in IBMX- or K^+-stimulated conditions. In the presence of IBMX (1 mM) and 2 mM K^+, thioperamide produced a dose-dependent increase of [^{3}H]histamine synthesis. The maximum potentiation by thioperamide of IBMX effects was about 47% compared with IBMX stimulation (Fig. 5). Similarly, thioperamide produced a dose-dependent increase of histamine synthesis in depolarizing conditions (30 mM K^+ and no IBMX). The maximal increase was of 45% at a concentration of 100 nM thioperamide, compared with K^+ stimulation (Fig. 5).

***R*p-cAMPS Prevents Thioperamide Effects in IBMX- and K^+-Stimulated Histamine Synthesis.** To confirm whether the effect of thioperamide in stimulated conditions (presence of IBMX or K^+) was mediated through AC-PKA pathway, we studied whether the PKA inhibitor *R*p-cAMPS could prevent the effect of the inverse agonist. Pretreatment with *R*p-cAMPS (10 μM) completely blocked the stimulatory effect of thioperamide (100 nM) on IBMX-stimulated samples (Fig. 6). Higher concentrations of the PKA inhibitor (100 and 500 μM) also decreased the stimulatory effect produced by IBMX. In depolarizing conditions (30 mM K^+), the effect of thioperamide (100 nM) was also prevented by 10 μM *R*p-cAMPS (Fig. 6). In contrast to IBMX, higher concentrations of the PKA inhibitor (100 and 500 μM) were unable to decrease the effect produced by K^+.

PKI_{14-22} Prevents Clobenpropit-Stimulated Histamine Synthesis in K^+-Depolarized Samples. To further confirm the specificity of the effects observed in Fig. 6, we looked at the effects on histamine synthesis of a different histamine H_3 antagonist (clobenpropit; Kathmann et al., 1993) and a different cAMP-dependent protein kinase inhibitor (PKI_{14-22}; Glass et al., 1989). As expected, clobenpropit

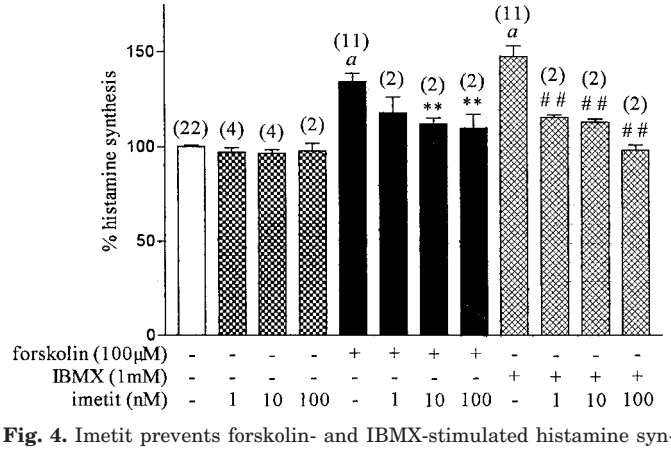

Fig. 4. Imetit prevents forskolin- and IBMX-stimulated histamine synthesis. Imetit was added to the medium 5 min before the addition of forskolin or IBMX to rat cerebral cortex miniprisms. Results are means ± S.E.M. Number of replicates is indicated in brackets above the columns. *a*, $P < 0.01$ compared with controls (2 mM K^+). **, $P < 0.01$ compared with forskolin stimulation. ##, $P < 0.01$ compared with IBMX-stimulated [^{3}H]histamine synthesis samples. 100% [^{3}H]histamine synthesis in the presence of 2 mM K+ and without any drug represents 21 ± 0.8 fmol of HA/mg of protein/h.

(10 nM) stimulated histamine synthesis in the presence of 30 mM K^+ (+59% compared with 30 mM K^+ stimulation) (Fig. 7). Similarly to thioperamide, clobenpropit had no effects in the absence of depolarization by K^+. Finally, PKI_{14-22} (10 μM) prevented clobenpropit potentiation of histamine synthesis in K^+-depolarized samples (−72% compared with clobenpropit stimulation) (Fig. 7).

Discussion

This is the first study of the signal transduction pathway used by H_3 autoreceptors to regulate histamine synthesis in histaminergic neurons. The recent cloning of histamine H_3 receptors by Lovenberg et al. (1999) showed that H_3 receptor agonists decreased cAMP accumulation elicited by forskolin stimulation of adenylate cyclase in recombinant receptor-transfected cells. Taking into account that H_3 receptor agonists inhibit histamine synthesis (Arrang et al., 1983) and that the histamine-synthesizing enzyme (histidine decarboxylase) has PKA phosphorylation sites (Joseph et al., 1990), it would be easy to hypothesize a connection among H_3 receptors, the AC-PKA pathway and histamine synthesis. Nevertheless, this fact had never been demonstrated. For these reason, the goals of this study were 1) to study the involvement of AC-PKA pathway on the histamine synthesis and 2) to asses whether this is the signal transduction mechanism used by H_3 autoreceptors to regulate histamine synthesis.

The present study clearly demonstrates that the adenylate cyclase-PKA pathway modulates histamine synthesis. This conclusion is based on results obtained with different kinds of drugs: [^{3}H]histamine synthesis was increased by forskolin (directly activating AC), db-cAMP (directly activating PKA), or IBMX (increasing intracellular cAMP levels through inhibition of phosphodiesterases) treatments. On the other hand, the PKA inhibitor *R*p-cAMPS prevented forskolin- and db-cAMP plus IBMX-stimulated histamine synthesis. Because *R*p-cAMPS is a highly specific PKA inhibitor (Botelho et al., 1988; Wang et al., 1991), all these results demonstrate that the increment of cAMP levels and the subsequent activation of PKA stimulates histamine synthesis. Nevertheless, further studies are necessary to clarify whether PKA-stimulated histamine synthesis is mediated through a direct phosphorylation of histidine decarboxylase into the PKA phosphorylation consensus sites described by Joseph et al. (1990). Alternatively, an indirect effect of PKA through modulation of other protein kinase cascades could also be possible.

In apparent disagreement with our data, results published previously by Huszti et al. (1991) suggested that PKA should negatively modulate histidine decarboxylase. In their work, the activity of crude histidine decarboxylase obtained from hypothalamus and lung was decreased by phosphorylating conditions. However, their results are difficult to interpret because of the low selectivity of the conditions used. In addi-

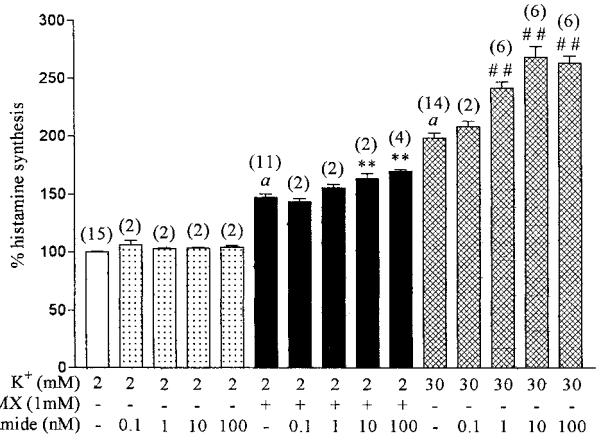

Fig. 5. The stimulatory effect of thioperamide on IBMX- or K^+-enhanced histamine synthesis. IBMX and thioperamide were added to the incubation of rat brain cortical miniprisms 15 min before the incubation period. Results represent means ± S.E.M. Number of replicates is indicated in brackets above the columns. *a*, $P < 0.01$ compared with 2 mM K^+, no drugs. *, $P < 0.05$, **, $P < 0.01$ compared with IBMX stimulation. ##, $P < 0.01$ compared with stimulation produced by 30 mM K^+. 100% [^{3}H]histamine synthesis in the presence of 2 mM K^+ and without drugs was 23 ± 0.6 fmol of HA/mg of protein/h.

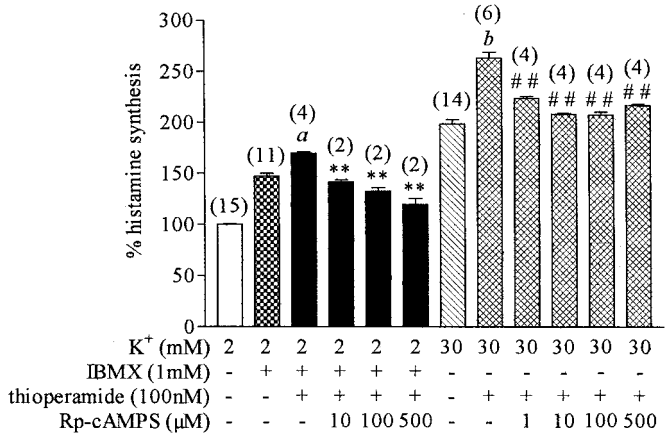

Fig. 6. The PKA inhibitor *R*p-cAMPS prevents thioperamide stimulated [^{3}H]histamine synthesis in the presence of IBMX or K^+. *R*p-cAMPS was added to the medium 5 min before thioperamide and IBMX addition to cerebral cortical miniprisms. The other drugs were added to the medium as indicated in Fig. 5. Results represent means ± S.E.M. Number of replicates is indicated in brackets above the columns. *a*, $P < 0.01$ compared with IBMX stimulation. **, $P < 0.01$ compared with IBMX plus thioperamide cotreatment. *b*, $P < 0.01$ compared with K^+ stimulation. ##, $P < 0.01$ compared with K^+ plus thioperamide cotreatment. 100% [^{3}H]histamine synthesis was 23 ± 0.5 fmol of HA/mg of protein/h in 2 mM K^+ with no drugs addition. Note that at 500 μM, *R*p-cAMPS also decreases IBMX effects, but not K^+ effects.

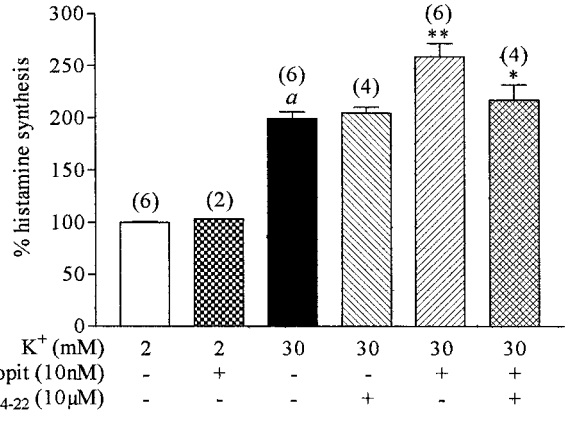

Fig. 7. Histamine synthesis stimulation by clobenpropit in the presence of 30 mM K^+ is impaired by PKI_{14-22}. PKI_{14-22} was added to the medium 5 min before clobenpropit addition to cerebral cortical miniprisms. Results represent means ± S.E.M. Number of replicates is indicated in brackets above the columns. *a*, $P < 0.01$ compared with 2 mM K^+, no drugs. **, $P < 0.01$ comparison with 30 mM K^+. *, $P < 0.05$ compared with clobenpropit in 30 mM K^+. 100% of histamine synthesis was 24.2 ± 0.2 fmol of HA/mg of protein/h in 2 mM K^+ without drugs addition.

tion, their results might not be mediated by PKA, because the addition or omission of exogenous PKA into the phosphorylation medium had no significant effects.

Once we demonstrated that the AC-PKA pathway modulates histamine synthesis, we wanted to clarify whether native H_3 autoreceptors regulate histamine synthesis through this cascade. As hypothesized, the H_3 agonist imetit prevented both forskolin- and IBMX-stimulated [^{3}H]histamine synthesis. Conversely, the H_3 inverse agonist thioperamide potentiated the effects of IBMX. Furthermore, in depolarizing conditions, the effects of two H_3 antagonists/inverse agonists (clobenpropit and thioperamide) were blocked by different PKA inhibitors. This clearly confirms that histamine H_3 receptors use the AC-PKA pathway to modulate histamine synthesis. A similar mechanism has been described for the regulation of the tyrosine hydroxylase activity by dopamine D_2 autoreceptors (El Mestikawy et al., 1985). In this case, D_2 autoreceptors are negatively coupled to AC modulating intracellular cAMP levels and PKA activity, which subsequently regulates tyrosine hydroxylase through direct phosphorylation (Arita and Kimura, 1986; Salah et al., 1989).

High forskolin concentrations may also have non-AC mediated effects (Laurenza et al., 1989), such as the modulation of several ion channels. Part of the forskolin effects we observed may not be related to AC. This would explain why *R*p-cAMPS and imetit prevented forskolin stimulation only partially. In contrast, *R*p-cAMPS and imetit completely impaired db-cAMP and IBMX effects. Thus, our results are strengthened by the fact that different drugs stimulated histamine synthesis acting at different levels of the AC-PKA pathway (H_3 receptors, AC, PKA, and cAMP degradation). Furthermore, different compounds inactivating the pathway at two levels (acting on H_3 receptors or blocking PKA) reversed these effects.

H_3 ligands did not modify basal (2 mM K^+) histamine synthesis. However, they had effects in the presence of AC-PKA stimulators (forskolin, IBMX) or in depolarized conditions (30 mM K^+). This suggests that histamine synthesis needs to be stimulated to be regulated by H_3 receptors. At present, we do not know what mechanisms are involved in K^+-stimulation of histamine synthesis. Thus, further work will be necessary to assess whether K^+-stimulation and AC-PKA activation converge at some point on histamine synthesis regulation.

The observation that H_3 receptors use the AC-PKA pathway to exert their function on neurotransmitter synthesis in histaminergic neurons does not exclude the possibility that additional signal transduction mechanisms are involved in this or other H_3 receptor-mediated events. Several reports describe an interaction of H_3 receptors with calcium related processes. For instance, Takeshita et al. (1998) reported that H_3 receptor could modulate voltage-sensitive calcium channels in neurons dissociated from the rat tuberomamillary nucleus. Schlicker et al. (1994) indicated that H_3 receptor-mediated inhibition of noradrenaline release was dependent of Ca^{2+} influx. Also, Blandizzi et al. (2001) suggested that H_3 receptor-inhibition of intestinal acetylcholine release could be mediated by modulation of N-type calcium channels. A direct coupling of G protein $\beta\gamma$ complexes to Ca^{2+} channels might produce these effects of H_3 receptors on voltage-dependent calcium channels (Diverse-Pierluissi et al., 1997 and 2000). It has also been described that H_3 receptors can modulate the effects of a calcium ionophore on arachidonic acid release (Morisset et al., 2000). On the other hand, other reports of signal transduction mechanisms related to H_3 receptors have been published. Cherify et al. (1992) described a negative coupling of histamine H_3 receptor to phospholipase C in human intestinal HGT-1 cells. Finally, Drutel et al. (2001) reported that activation of several rat H_3 receptor isoforms led to activation of the MAP kinase signaling cascade via pertussis toxin-sensitive G proteins. Several H_3 receptor isoforms varying in the length of their third intracellular loops have been identified (Tardivel-Lacombe et al., 2000; Drutel et al., 2001). Because this is the molecular domain thought to be responsible for coupling to G proteins, it is possible that different H_3 isoforms have differences in their signaling pathways. It should be mentioned that we have obtained results on the involvement of AC-PKA pathway in histamine synthesis in the absence of depolarization. This fact gives more consistency to our conclusions, because the effects observed in depolarizing conditions involve general neurotransmitters release, which could complicate the interpretation of the results observed.

In conclusion, we have demonstrated that brain histamine synthesis can be stimulated by activators of cAMP-dependent protein kinase. Subsequently, we have shown that this protein kinase participates in the H_3 receptor transduction mechanism modulating histamine synthesis. However, the involvement of other signaling pathways can not be excluded.

References

Arita J and Kimura F (1986) Adenosine 3′,5′-cyclic monophosphate stimulates dopamine biosynthesis in the median eminence of rat hypothalamic slices. *Brain Res* **374:**37–44.

Arrang JM, Garbarg M, Lancelot JC, Lecomte JM, Pollard H, Robba M, SchunakW and Schwartz JC (1987a) Highly potent and selective ligands for histamine H_3-receptors. *Nature (Lond)* **327:**117–123.

Arrang JM, Garbarg M, and Schwartz JC (1983) Auto-inhibition of brain histamine release mediated by a novel class (H_3) of histamine receptor. *Nature (Lond)* **302:**832–837.

Arrang JM, Garbarg M, and Schwartz JC (1987b) Autoinhibition of histamine synthesis mediated by presynaptic H_3-receptors. *Neuroscience* **23:**149–157.

Ash ASF and Schild HO (1966) Receptors mediating some actions of histamine. *Br J Pharmacol* **27:**427–439.

Beavo JA and Reifsnyder DH (1990) Primary sequence of cyclic nucleotide phosphodiesterase isozymes and the design of selective inhibitors. *Trends Pharmacol Sci* **11:**150–155.

Black JW, Duncan WAM, Durant CJ, Ganellin CR, and Parsons EM (1972) Definition and antagonism of histamine H2-receptors. *Nature (Lond)* **236:**385–390.

Blandizzi C, Colucci R, Tognetti M, De Paolis B, and Del Tacca M (2001) H_3-receptor-mediated inhibition of intestinal acetylcholine release: pharmacological characterization of signal transduction pathways. *Naunyn-Schmiedeberg's Arch Pharmacol* **363:**193–202.

Botelho LHP, Rothermel JD, Coombs RV, and Jastorff B (1988) cAMP analog antagonists of cAMP action. *Methods Enzymol* **159:**159–172.

Cherify Y, Pigeon C, Le Romancer M, Bado A, Reyl-Desmars F, and Lewin MJM (1992) Purification of a histamine H_3 receptor negatively coupled to phosphoinositide turnover in the human gastric cell line HGT1. *J Biol Chem* **267:**25315–25320.

Clark EA and Hill SJ (1996) Sensitivity of histamine H_3 receptor agonist-stimulated [^{35}S]GTPγ[S] binding to pertussis toxin. *Eur J Pharmacol* **296:**223–225.

Daly JW, Padgett W, and Seamon KB (1982) Activation of cyclic AMP-generating systems in brain membranes and slices by the diterpene forskolin: augmentation of receptor-mediated responses. *J Neurochem* **38:**532–544.

Diverse-Pierluissi M, McInteire WE, Myung CS, Lindorfer MA, Garrison JC, Goy MF and Dunlap K (2000) Selective coupling of G-protein $\beta\gamma$ complexes to inhibition of Ca^{2+} channels. *J Biol Chem* **275:**28380–28385.

Diverse-Pierluissi M, Remmers AE, Neubig RR, and Dunlap K (1997) Novel form of crosstalk between G protein and tyrosine kinase pathway. *Proc Natl Acad Sci USA* **94:**5417–5421.

Drutel G, Peitsaro N, Karlstedt K, Wieland K, Smit MJ, Timmerman H, Panula P, and Leurs R (2001) Identification of rat H_3 receptor isoforms with different brain expression and signaling properties. *Mol Pharmacol* **59:**1–8.

El Mestikawy S, Gozlan H, Glowinski J, and Hamon M (1985) Characteristics of tyrosine hydroxylase activation by K^+-induced depolarization and/or forskolin in rat striatal slices. *J Neurochem* **45(1):**173–184.

Garbarg M, Arrang JM, Rouleau A, Ligneau X, Dam Trung Tuong M, and Schwartz JC (1992) *S*-[2-(4-Imidazolyl)ethyl]Isothiourea, a highly specific and potent histamine H_3 receptor agonist. *J Pharmacol Exp Ther* **263:**304–310.

Glass DB, Cheng HC, Mende-Mueller L, Reed J, and Walsh DA (1989) Primary structural determinants essential for potent inhibition of cAMP-dependent protein kinase by inhibitory peptides corresponding to the active portion of the heat-stable inhibitor protein. *J Biol Chem* **264:**8802–8810.

Huszti Z, Magyar K, and Keleti J (1991) Possible regulation of hypothalamus and lung histidine decarboxylase activity by cAMP-dependent protein kinase. *Eur J Biochem* **197:**191–196.

Joseph DR, Sullivan PM, Wang Y-M, Kozak C, Fenstermacher DA, Behrendsen ME, and Zahnow CA (1990) Characterization and expression of the complementary DNA encoding rat histidine decarboxylase. *Proc Natl Acad Sci USA* **87:**733–737.

Kathman M, Schlicker E, Detzner M, and Timmerman H (1993) Nordimaprit, homodimaprit, clobenpropit, and imetit: Affinities for H_3 binding sites and potencies in a functional H_3 receptor model. *Naunyn-Schmiederg's Arch Pharmacol* **348:**498–503.

Laurenza A, McHugh Sutkowski E, and Seamon KB (1989) Forskolin: a specific stimulator of adenylyl cyclase or a diterpene with multiple sites of action? *Trends Pharmacol Sci* **10:**442–447.

Lovenberg TW, Pyati J, Chang H, Wilson SJ, and Erlander MG (2000) Cloning of rat histamine H_3 receptor reveals distinct species pharmacological profiles. *J Pharmacol Exp Ther* **293:**771–778.

Lovenberg TW, Roland BL, Wilson SJ, Jiang X, Pyati J, Huvar A, Jackson MR, and Erlander M (1999) Cloning and functional expression of the human histamine H_3 receptor. *Mol Pharmacol* **55:**1101–1107.

Lowry OH, Rosebrough NJ, Farr AL, and Randall RJ (1951) Protein measurements with the Folin phenol reagent. *J Biol Chem* **193:**265–275.

Morgan AJ, Murray KJ, and Challiss RAJ (1993) Comparison of the Effect of Isobutylmethylxanthine and Phosphodiesterase-Selective Inhibitors on cAMP levels in SH-SY5Y Neuroblastoma Cells. *Biochem Pharmacol* **45:**2373–2380.

Morisset S, Rouleau A, Ligneau X, Gbahou F, Tardivel-Lacombe J, Stark H, Schunack W, Ganellin CR, Schwartz JC, and Arrang JM (2000) High constitutive activity of native H_3 receptors regulates histamine neurons in brain. *Nature (Lond)* **408:**860–864.

Ortiz J, Gomez-Ramirez J, Torrent A, Aldavert M, and Blanco I (2000) Quantitative radioisotopic determination of histidine decarboxylase using high-performance liquid chromatography *Anal Biochem* **280:**111–117.

Posternak T and Weiman G (1974) The preparation of acylated derivatives of cyclic nucleotides. *Methods Enzymol* **38:**399–409.

Salah RS, Kuhn DB, and Galloway MP (1989) Dopamine autoreceptors modulate the phosphorylation of tyrosine hydroxylase in rat striatal slices. *J Neurochem* **52:** 1517–1522.

Schlicker E, Kathman M, Dtezner M, Exner HM, and Gother M (1994) H_3 receptor-mediated inhibition of noradrenaline release: an investigation into the involvement of Ca^{2+} and K^+ ions, G protein and adenylate cyclase. *Naunyn-Schmiedeberg's Arch Pharmcol* **350:**34–41.

Schwartz JC, Arrang JM, Garbarg M, Pollard H, and Ruat M (1991) Histaminergic transmission in the mammalian brain. *Physiol Rev* **71:**1–51.

Seamon KB, Daly JW, Metzger H, de Souza NJ, and Reden J (1983) Structure-activity relationships for activation of adenylate cyclase by the diterpene forskolin and its derivatives. *J Med Chem* **26:**436–439.

Takeshita Y, Watanabe T, Sakata T, Munakata M, Ishibashi H, and Akaike N (1998) Histamine modulates high-voltage-activated calcium channels in neurons dissociated from the rat tuberomammillary nucleus. *Neuroscience* **87:**797–805.

Tardivel-Lacombe J, Rouleau A, Heron A, Morisset S, Pillot C, Cochois V, Schwartz JC, and Arrang JM (2000) Cloning and cerebral expression of the guinea pig histamine H_3 receptor: evidence for two isoforms. *Neuroreport* **11:**755–759.

Wang L-Y, Salter MW, and MacDonald JF (1991) Regulation of Kainate receptors by cAMP-dependent protein kinase and phosphatases. *Science (Wash DC)* **253:**1132–1135.

Address correspondence to: Jordi Ortiz, Ph.D., Dept. Biochemistry and Molecular Biology, School of Medicine, Universitat Autònoma de Barcelona, E-08193, Spain. E-mail: jordi.ortiz@uab.es