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MOLECULAR PHARMACOLOGY

# H$_3$ Autoreceptors Modulate Histamine Synthesis through Calcium/Calmodulin- and cAMP-Dependent Protein Kinase Pathways

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## ABSTRACT

H$_3$ autoreceptors provide feedback control of neurotransmitter synthesis in histaminergic neurons, but the transduction pathways involved are poorly understood. In rat brain cortical slices, histamine synthesis can be stimulated by depolarization and inhibited by H$_3$ agonists. We show that histamine synthesis stimulation by depolarization with 30 mM K$^+$ requires extracellular calcium entry, mostly through N-type channels, and subsequent activation of calcium/calmodulin-dependent protein kinase type II. In vitro, this kinase phosphorylated and activated histidine decarboxylase, the histamine-synthesizing enzyme. Inhibition of depolarization-stimulated histamine synthesis by the histamine H$_3$ receptor agonist imetit was impaired by preincubation with pertussis toxin and by the presence of a myristoylated peptide (myristoyl-N-QEHAQEPERQYMHIGTMVE-FAYALVGK) blocking the actions of G-protein $\beta\gamma$ subunits. The stimulation of another G$_{i/o}$-coupled receptor, adenosine A$_1$, also decreased depolarization-stimulated histamine synthesis. In contrast, protein kinase A activation, which is also repressed by H$_3$ receptors, elicited a depolarization- and calcium/calmodulin-independent stimulation of histamine synthesis. Protein kinase A was able also to phosphorylate and activate histidine decarboxylase in vitro. These results show how depolarization activates histamine synthesis in nerve endings and demonstrate that both pathways modulating neurotransmitter synthesis are controlled by H$_3$ autoreceptors.

Histaminergic neurons modulate several physiological processes, such as sleep/wake cycles, food intake, and memory (Brown et al., 2001). Four subtypes of histamine receptors (H$_1$ to H$_4$) have been identified and cloned. The H$_3$ receptor is the only subtype present in histaminergic neurons, in which it mediates inhibition of histamine release and synthesis, functioning as a classic autoreceptor (Arrang et al., 1983, 1987). H$_3$ receptors are also expressed by other neurons, mostly cortical, thalamic, and striatal projection neurons (Pillot et al., 2002). Structurally, H$_3$ receptors are members of the seven transmembrane receptor superfamily (Lovenberg et al., 1999) and couple to G$_{i/o}$ proteins (Clark and Hill, 1996). Stimulation of H$_3$ receptors decreases extracellular calcium inflow in neurons (Takeshita et al., 1998; Blandizzi et al., 2001), inhibits adenylate cyclase (Lovenberg et al., 1999; Gomez-Ramirez et al., 2002), and increases mitogen-activated protein kinase phosphorylation in receptor-transfected cells (Drutel et al., 2001). It has been shown that H$_3$ autoreceptors have significant constitutive activity, resulting in partial tonic autoinhibition of histaminergic neurons in the absence of histamine (Morisset et al., 2000).

Despite the potential usefulness and the many pharmacological effects described for H$_3$ receptor ligands (Brown et al., 2001), little is known about the transduction mechanisms triggered by H$_3$ receptors within histaminergic neurons. These neurons are characterized by the presence of the en-

**ABBREVIATIONS:** HDC, histidine decarboxylase; AIP, myristoylated autocamtide-2-related inhibitory peptide; CaMKII, calcium/calmodulin-dependent protein kinase type II; IBMX, 1-methyl-3-isobutylxanthine; KN-62, 1-[N,O-bis(5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine; PKA, protein kinase A; PKI$_{14-22}$ = myristoylated protein kinase A inhibitor amide 14-22; Myr-QEHA peptide, myristoyl-N-QEHAQEPERQYMHIGTMVE-FAYALVGK; W-13 = N-(4-aminobutyl)-5-chloro-2-naphthalenesulfonamide; HPLC, high-performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; ANOVA, analysis of variance.

zyme-synthesizing histamine histidine decarboxylase (HDC). HDC is a pyridoxal-5′-phosphate–dependent decarboxylase translated as a 74-kDa precursor that undergoes carboxyl-terminal truncation to yield the active forms of the enzyme (Joseph et al., 1990; Dartsch et al., 1999; Fleming and Wang, 2003; Rodriguez-Caso et al., 2003). In a previous work, we showed that histamine synthesis can be stimulated by the adenylate cyclase/protein kinase A pathway in nerve endings, an effect that is inhibited by $H_3$ receptors (Gomez-Ramirez et al., 2002). Because protein kinase A (PKA) activation was also required for the effects of $H_3$ receptor inverse agonists, we concluded that this pathway mediated $H_3$ auto-receptor inhibition of histamine synthesis. However, we also showed that histamine synthesis can be stimulated by depolarization with $K^+$ ions independently of PKA blockade (Gomez-Ramirez et al., 2002). Because $H_3$ agonists inhibit the effects of depolarization (Arrang et al., 1987), we hypothesize that $H_3$ receptors must be coupled to another transduction pathway modulating histamine synthesis besides adenylate cyclase/PKA. Our goal in this work was to investigate this supposed pathway. Here, we show that depolarization opens voltage-sensitive calcium channels and activates calcium/calmodulin-dependent protein kinase type II (CaMKII), leading to increased histamine synthesis. Therefore, both PKA and CaMKII can phosphorylate a recombinant version of catalytically active HDC in vitro, leading to increased activity. In brain slices, stimulation of $H_3$ receptors represses the effects elicited through either of these kinases, which apparently are mutually independent.

## Materials and Methods

**Chemicals.** Ring-labeled [2,5-$^3$H]L-histidine (50 Ci/mmol) obtained from Amersham Biosciences UK, Ltd. (Little Chalfont, Buckinghamshire, UK) was purified by high-performance liquid chromatography (HPLC) before use as described by Ortiz et al. (2000). $PKI_{14-22}$, 1-methyl-3-isobutylxanthine (IBMX), KN-62, W-13, myristoylated autocamtide-2–related inhibitory peptide (AIP), pertussis toxin, calmodulin, and CaMKII were obtained from Merck Biosciences (Darmstadt, Germany). ω-Conotoxins MVIIC and GVIA were purchased from Tocris Cookson Inc. (Bristol, UK). The $\beta\gamma$ inhibitor 27-mer QEHA peptide (Chen et al., 1995) was synthesized and N-terminal–myristoylated by Pepscan (Lelystad, Netherlands). Imetit, HPLC standards, and other reagents were purchased from Sigma/RBI (Steinheim, Germany). OptiPhase HiSafe-3 liquid scintillation cocktail was from PerkinElmer Wallac (Turku, Findland).

**Preparation and Incubation of Brain Slices.** Male Sprague-Dawley rats weighing 200 to 250 g (Animal Service, Universitat Autònoma de Barcelona, Barcelona, Spain) were killed by decapitation between 10 and 11 AM. Brains were chilled immediately in modified Krebs-Ringer-bicarbonate medium with the following 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), cortical lobes were dissected and sliced in a McIlwain tissue chopper to obtain miniprisms of $0.3 \times 0.3$ mm/side. The miniprisms were suspended in ice-cold Krebs-Ringer-bicarbonate medium and washed to remove debris of damaged cells. Aliquots (100 $\mu$l) of the settled slice suspension were pipetted into 2-ml polypropylene tubes. Up to 24 tubes containing slices were obtained from a single rat brain cortex. The tubes were preincubated for 25 min at 37°C in an Eppendorf Thermomixer under 95% $O_2$/5% $CO_2$ atmosphere (5 Prime, Inc., Boulder, CO). Previously purified [$^3$H]histidine was added to all samples (6.25 $\mu$Ci/tube, to a final concentration of 0.5 $\mu$M), and the tubes were incubated for 5 min to allow [$^3$H]histidine uptake. If depolarizing

conditions were required, buffer was added with concentrated KCl up to a total volume of 250 $\mu$l. NaCl concentration in depolarizing buffer was decreased proportionally to maintain isotonicity. Miniprisms were then incubated for 30 min to synthesize [$^3$H]histamine. When drugs were tested ($H_3$ ligands, phosphodiesterase, or kinase inhibitors, calcium-channel blockers), they were added 15 or 20 min before depolarization. Several drug concentrations were routinely tested, because diffusion across the slices may be very different between compounds. This is especially important for protein kinase inhibitors, because relatively high concentrations were needed to achieve intracellular effects (Fig. 2). To stop incubations, the tubes were placed on ice, and immediately trichloroacetic acid was added (final concentration, 1%) containing 100 nmol histamine per tube (internal standard). Blank samples were added to trichloroacetic acid before [$^3$H]histidine, and they were stored on ice during incubation of the rest of samples. All samples were homogenized in a Dynatech/Sonic Dismembrator at 4°C (Dynatech Labs, Chantilly, VA). An aliquot was taken for protein quantification by the Lowry method to take into account the variability of tissue amounts contained into each tube (usually 2–3 mg protein per tube, using bovine serum albumin as standard). Samples were then centrifuged (12,000$g$, 10 min, 4°C), and supernatants were recovered and processed through [$^3$H]histamine purification by HPLC.

**[$^3$H]Histamine Purification by HPLC.** Deproteinized supernatants were mixed with 100 $\mu$l of ion-exchange resin (Amberlite IRA 900 mesh 16-50; Supelco, Bellefonte, PA) prepared as described by Ortiz et al. (2000) and vortexed for 10 min to allow histidine binding. The tubes were centrifuged, and the supernatants recovered. This process eliminates 86% [$^3$H]histidine, increasing the sensitivity of subsequent [$^3$H]histamine purification. The supernatants were injected into a Merck-Hitachi HPLC system equipped with an L-7200 autosampler and a reverse-phase C18 column ($25 \times 0.46$-cm Tracer Extrasil ODS-2, 5 $\mu$m particle size; Teknokroma, Barcelona, Spain) with $2 \times 20$-mm guard column (Upchurch Scientific, Oak Harbor, WA). The mobile phase was made of 21% (v/v) methanol, 10 mM octanesulfonic acid ion pair, and 0.3 M sodium phosphate buffer, adjusted to pH 3. Histamine eluted isocratically at 10 to 11 min with 1 ml/min flow rate. The histamine internal standard was the main peak apparent in all samples using ultraviolet detection at 225 nm. Endogenous histamine and formed [$^3$H]histamine were not in sufficient amounts to be detected by ultraviolet detection. Histamine internal standard peak detection automatically started a fraction collector (Merck-Hitachi L-5200) recovering eluted histamine. Collected histamine fractions were mixed with Optiphase scintillation cocktail, and disintegrations per minute were counted. Recovery (typically approximately 75%) was estimated for each sample by comparing internal- and external-standard histamine peak areas detected by ultraviolet detection and quantified using a Hercule 2000 interface with Borwin software (JMBS, Grenoble, France). [$^3$H]Histamine synthesis in each sample was estimated from disintegrations per minute obtained corrected by recovery, blank disintegrations per minute, and specific activity of [$^3$H]histidine and then expressed as a function of protein content and incubation time.

**Expression and Purification of Recombinant HDC.** A cDNA encoding the 1/512 N-terminal sequence of rat HDC was obtained by reverse transcriptase-polymerase chain reaction from fetal rat liver as described by Engel et al. (1996). The cDNA was digested with BamHI and ligated into a pET-11a vector (Novagen, Madison, WI), as described by Olmo et al. (2002). The vector was expressed in BL21(DE3)pLysS-competent *Escherichia coli* cells by induction with isopropyl thiogalactoside following the manufacturer's instructions. The cells were pelleted by centrifugation, resuspended in 20 mM potassium buffer, pH 7.0, containing 2 mM EDTA, 5 $\mu$M pyridoxal-5′-phosphate, and 1% polyethylene glycol 300, and lysed by sonication. The lysate was centrifuged for 90 min at 14,000 rpm at 4°C, and the supernatant was recovered for purification of HDC protein. Purification was performed according to the procedure described by Olmo et al. (2002). All purification steps were monitored by SDS-

PAGE and Western blot using an anti-HDC antibody (Dartsch and Persson, 1998). In brief, ammonium sulfate was added to saturation, and the precipitates were discarded. The supernatants were subjected to phenyl Sepharose chromatography (CL-4B; Pfizer, Inc., Täby, Sweden) and eluted with buffer containing 1 mM potassium phosphate, pH 7.0, 0.1 mM dithiothreitol, 5 $\mu$M pyridoxal-5′-phosphate, and 1% polyethylene glycol 300. The eluates were concentrated by ultrafiltration in Amicon cells (Millipore Corporation, Bedford, MA) and subjected to DEAE interchange chromatography (DEAE-Toyopearl column 650M; TOSOH, Tokyo, Japan) using a linear NaCl gradient in the previous buffer. HDC eluted at 0.1 M NaCl. Finally, hydroxyapatite chromatography (Bio-Rad, Hercules, CA) was used in a potassium phosphate gradient increasing up to 200 mM phosphate. Both by Western blot and Coomassie blue staining, the purified fraction yielded a single band at $M_r$ 55,000, the expected size of 1/512 HDC.

**Phosphorylation and Activity of Recombinant HDC.** For CaMKII phosphorylation, 30 $\mu$g of purified HDC was incubated in 50 mM HEPES buffer, pH 7.4, in the presence of 60 ng of CaMKII (Calbiochem, San Diego, CA), 3 $\mu$g of calmodulin (Calbiochem), 0.3 mM CaCl₂, 5 mM MgCl₂, and 50 $\mu$M ATP (containing 10 $\mu$Ci [$\gamma$-³²P]ATP; Amersham Biosciences) in a total volume of 100 $\mu$l. Incubations were carried out for 40 min at 30°C and then stopped by the addition of SDS-PAGE sample buffer. Control samples were incubated without CaMKII. For PKA phosphorylation, the same conditions were used, except that 10 $\mu$g of purified HDC were incubated with 0.1 $\mu$g PKA (catalytic subunit from bovine heart; Sigma/RBI). Calmodulin and CaCl₂ were omitted. Samples were boiled for 5 min, and 10 $\mu$l was subjected to SDS-PAGE in gels containing 10% polyacrylamide. Molecular weight markers used were Bio-Rad Precision Plus. After Coomassie blue stain, the gels were dried over vacuum for 30 min at 70°C and exposed to Kodak X-omat films using amplifying screens. Radiolabeled bands were quantified by densitometry using Scion Image software (Scion Corporation, Frederick, MD). To determine changes in HDC activity, 0.45 $\mu$g of HDC was phosphorylated in the above conditions, except that [$\gamma$-³²P]ATP was omitted. Thereafter, phosphorylated HDC and control samples were incubated in 50 mM HEPES buffer, pH 7.4, 5 mM MgCl₂, 10 $\mu$M pyridoxal-5′-phosphate, and 60 $\mu$M histidine (0.1 $\mu$M [³H]histidine of 43 Ci/mmol) in a final volume of 100 $\mu$l. Samples were incubated for 30 min at 37°C, and [³H]histamine formation was quantified as described by Ortiz et al. (2000).

**Statistical Analysis.** Because the number of slice incubations in one experiment is limited to a maximum of 24 (see *Preparation and Incubation of Brain Slices*), data from different experiments were normalized to the percentage of basal histamine synthesis and pooled. All experiments were balanced with the appropriate controls. Statistical significance of differences between values was evaluated by analysis of variance (ANOVA) followed by Bonferroni post hoc tests for multiple-group comparisons.

## Results

In preliminary experiments, we observed that histamine synthesis in rat brain cortical slices could be stimulated by depolarization with either K⁺ or 4-aminopyridine in a dose-dependent manner. Maximal effects of these two agents were not additive, suggesting that they were acting on the same mechanisms (data not shown). To test whether extracellular calcium inflow was required, we incubated slices in the presence of 0.2 mM cadmium, a nonselective inhibitor of voltage-sensitive calcium channels. Cadmium significantly decreased the effects of depolarization with 30 mM K⁺ by 66% (data not shown). In following experiments, we used channel-selective inhibitor toxins. Elsewhere, 3 $\mu$M $\omega$-conotoxin MVIIC has been reported to completely block Q-, P-, and N-type calcium

currents (Hillyard et al., 1992; Takeshita et al., 1998). $\omega$-Conotoxin MVIIC at this concentration completely inhibited depolarization induction of histamine synthesis (Fig. 1), which suggests the requirement of calcium inflow through at least one of these channel subtypes. In contrast, no effect was observed when the same MVIIC toxin was used at a lower concentration (0.1 $\mu$M) capable of blocking only Q-type calcium currents in histaminergic neurons (Takeshita et al., 1998). The N-type selective $\omega$-conotoxin GVIA (Kerr and Yoshikami, 1984) blunted depolarization effects by 84% (Fig. 1). These results indicate that N-type calcium channels are the main gate of calcium inflow participating in the activation of histamine synthesis by KCl depolarization in histaminergic nerve endings. The P-type calcium channel seems to contribute to a minor extent.

Next, we sought a calcium-binding protein that could participate in the activation of histamine synthesis. A concentration-dependent inhibition of depolarization effects was obtained when the slices were incubated with the calcium/calmodulin inhibitor W-13 (Chafouleas et al., 1982) (Fig. 2A). Depolarization effects were almost completely suppressed (−92%) at the highest concentration of W-13 assayed (1 mM). This dose of W-13 may seem relatively high compared with the IC₅₀ of calmodulin inhibition in homogenates (approximately 60 $\mu$M) (Chafouleas et al., 1982); however, it must be considered that the molecule must diffuse through the dense tissue-slice preparation and cross-membranes to reach its intracellular target. Thereafter, we tested two different CaMKII inhibitors: KN-62 (Tokumitsu et al., 1990) and AIP (Ishida et al., 1995). Both agents prevented histamine synthesis stimulation by depolarization in a dose-dependent manner (Fig. 2, B and C). Maximal inhibition of depolarization effects was 93 and 91% for KN-62 and the inhibitor peptide, respectively. As expected, all of these compounds needed at least 1 order of magnitude higher concentrations when used in slices compared with their reported IC₅₀ values in homogenates, a difference that was even greater in the case of AIP, whose highly hydrophilic sequence should lead to difficult membrane crossing.

The previous results raised the question of whether CaMKII could directly phosphorylate the histamine synthe-
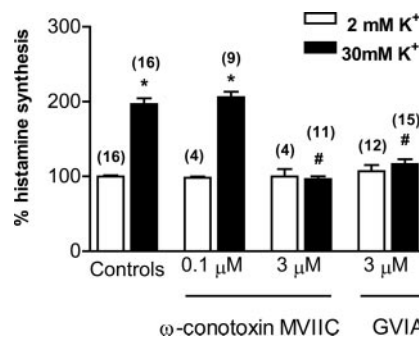


**Fig. 1.** Blockade of N- and P-type voltage-gated calcium channels prevents depolarization-induced histamine synthesis. Toxins were applied 15 min before incubation of rat brain cortical slices with [³H]histidine and depolarization with 30 mM KCl. $\omega$-Conotoxin MVIIC (3 $\mu$M) inhibits N-, Q-, and P-type calcium channels, whereas at 0.1 $\mu$M, it only inhibits Q-type channels. $\omega$-Conotoxin GVIA blocks N-type calcium channels. Results represent means ± S.E.M. Number of slice incubations are indicated between brackets above the columns. Mean 100% of histamine synthesis in 2 mM K⁺ controls was 18 fmol mg protein$^{-1}$ h$^{-1}$. ⋆, $p < 0.01$ compared with controls (2 mM K⁺); #, $p < 0.01$ compared with 30 mM K⁺, one-way ANOVA plus Bonferroni post hoc tests.

sizing enzyme HDC. Several potential phosphorylation sites could be predicted from the full HDC sequence obtained by Joseph et al. (1990) (http://www.cbs.dtu.dk/databases/PhosphoBase/predict/predform.html). The full-length HDC gene encodes a 74-kDa protein that requires posttranslational carboxyl-terminal truncation to be catalytically active (Joseph et al., 1990). We obtained purified HDC by bacterial expression of a plasmid encoding 512 amino acids of the N-terminal side of the rat enzyme (1/512 HDC), which yields a catalytically active protein with size and characteristics similar to those of native HDC (Taguchi et al., 1984; Engel et al., 1996; Olmo et al., 2002; Rodriguez-Caso et al., 2003). Incubation of purified recombinant HDC in the presence of commercial CaMKII, $Ca^{2+}$, calmodulin, and $[\gamma\text{-}^{32}P]ATP$ resulted in strong $^{32}P$ incorporation, as determined by autoradiography of vacuum-dried SDS-PAGE gels. We $^{32}P$-labeled a protein of $M_r$ 55,000, the expected size of 1/512 HDC (Fig. 3A, right lane). Incubation controls containing CaMKII, $Ca^{2+}$, calmodulin, and $[\gamma\text{-}^{32}P]ATP$ but lacking purified HDC showed a minor incor-

poration of $^{32}P$ into proteins of similar molecular mass (Fig. 3A, left lane), which is probably caused by the expected autophosphorylation of CaMKII $\alpha$ and $\beta$ subunits of 50 to 60 kDa (Lai et al., 1987). However, the relative optical density of CaMKII autophosphorylation bands was three times lower than those of HDC phosphorylation. Incubations without CaMKII were not different from background (Fig. 3A, middle lane).

To find out whether phosphorylation by CaMKII would activate HDC, we incubated previously phosphorylated recombinant 1/512 HDC with 60 $\mu$M histidine (0.1 $\mu$M [$^3$H]histidine) as described by Ortiz et al. (2000). As shown in Fig. 3B, phosphorylation by CaMKII resulted in a 78% increase in enzyme activity in vitro (Fig. 3B). The substrate concentration chosen is lower than the $K_m$ of the enzyme at this pH (Ortiz et al., 2000). Therefore, the changes observed could represent an increase in $V_{max}$ or a decreased $K_m$ for the substrate.

The most likely mechanism by which $H_3$ receptors could repress histamine synthesis activation by depolarization would be the inhibition of voltage-sensitive calcium channels by G protein $\beta\gamma$ subunits (Takeshita et al., 1998; Diverse-Pierluissi et al., 2000). To test whether the blockade of $\beta\gamma$ actions would prevent the effects of a histamine $H_3$ agonist, we incubated brain slices in the presence of different concentrations of a peptide (QEHA peptide) that binds to $\beta\gamma$ subunits, impairing their interaction with targets (Chen et al., 1995). The QEHA peptide has been shown previously to inhibit the actions of G protein $\beta\gamma$ subunits on several effectors such as adenylate cyclases, G protein-regulated kinase, and a $K^+$ channel (Chen et al., 1995). The peptide we used
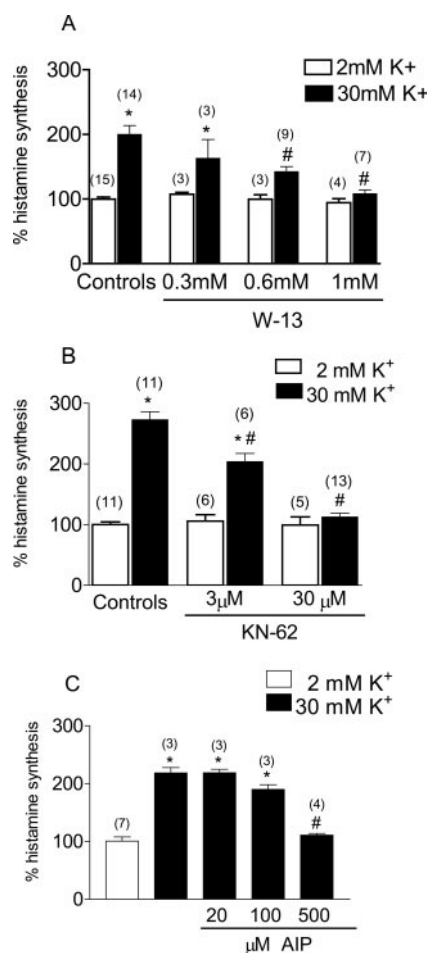


**Fig. 2.** Inhibition of CaMKII in rat brain cortical slices impairs depolarization-induced histamine synthesis. A, effect of the calmodulin antagonist W-13. B, effect of the CaMKII inhibitor KN-62. C, effect of the CaMKII inhibitor myristoyl-AIP. The inhibitors were applied at several concentrations to ensure sufficient intracellular diffusion; 15 min later, [$^3$H]histidine was added to the incubation buffer, and the slices were depolarized with 30 mM KCl. Results are means ± S.E.M. Number of slice incubations are indicated in brackets above the columns. Mean 100% of histamine synthesis in 2 mM K$^+$ controls was 17 fmol mg protein$^{-1}$ h$^{-1}$. ★, $p < 0.001$ compared with controls (2 mM K$^+$); #, $p < 0.001$ compared with 30 mM K$^+$, one- or two-way ANOVA plus Bonferroni post hoc test.
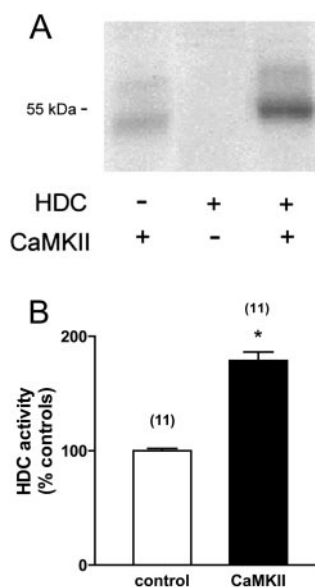


**Fig. 3.** Calcium/calmodulin-dependent protein kinase II phosphorylates and activates recombinant 1/512 HDC in vitro. A, autoradiogram showing the incorporation of $^{32}P$ into a protein of the expected molecular size of 1/512 HDC in the presence of CaMKII (right lane). Minor incorporation of $^{32}P$ into the kinase itself was expected as CaMKII subunits of 50 to 60 kDa undergo autophosphorylation (left lane). B, CaMKII-phosphorylated samples have higher histidine decarboxylase activity. Activity was determined in the presence of 60 $\mu$M histidine and 0.1 $\mu$M [$^3$H]histidine after CaMKII phosphorylation in vitro. Results are means ± S.E.M. Number of determinations is indicated in brackets above the columns. Mean 100% of HDC activity was 870 nmol mg protein$^{-1}$ h$^{-1}$. ★, $p < 0.001$ two-tailed Student's $t$ test.

was N-terminal–myristoylated to facilitate membrane crossing. Incubation of brain slices with the highest concentration of Myr-QEHA peptide blocked inhibition by imetit of depolarization-induced histamine synthesis by 79% (Fig. 4A). The peptide alone elicited a 19% increase in basal histamine synthesis (Fig. 4A). To confirm that G$_{i/o}$ protein subunits were involved in the effects of imetit, we incubated slices in the presence of 7.5 $\mu$g/ml pertussis toxin for 3 h. The use of pertussis toxin in tissue slices has the disadvantage that it requires long incubation times to be internalized. The 3-h incubation decreased histamine synthesis by 63%, probably because of the short half-life of histidine decarboxylase (Engel et al., 1996; Rodriguez-Caso et al., 2003). Nevertheless, because the slices at 3 h showed the normal effects of depolarization and imetit (Gomez-Ramirez et al., 2002), we could determine that pertussis toxin reduced the effects of imetit by 31% (Fig. 4B). To further analyze the G proteins partici-

pating in the inhibition of depolarization-induced calcium entry, we also incubated slices with several different G$_{i/o}$-coupled receptor agonists. We obtained no evidence of M$_{2/4}$ muscarinic, $\alpha_2$-adrenergic, GABA$_B$, or 5-hydroxytryptamine-1B serotonin receptors inhibiting depolarization-induced histamine synthesis (data not shown), perhaps because these receptors might not be present in the same nerve terminals. In contrast, the A$_1$-adenosine agonist $N^6$-cyclopentyladenosine did decrease dose-dependently depolarization-induced histamine synthesis, with a maximum of 33% inhibition. $\beta\gamma$ subunits should inhibit calcium channels (Takeshita et al., 1998; Diverse-Pierluissi et al., 2000), although additional actions on adenylate cyclases could not be discarded. Because histamine H$_3$ receptors can also modulate the adenylate cyclase/PKA pathway (Lovenberg et al., 1999; Gomez-Ramirez et al., 2002) and PKA can phosphorylate some calcium channels (Rotman et al., 1995), we tested
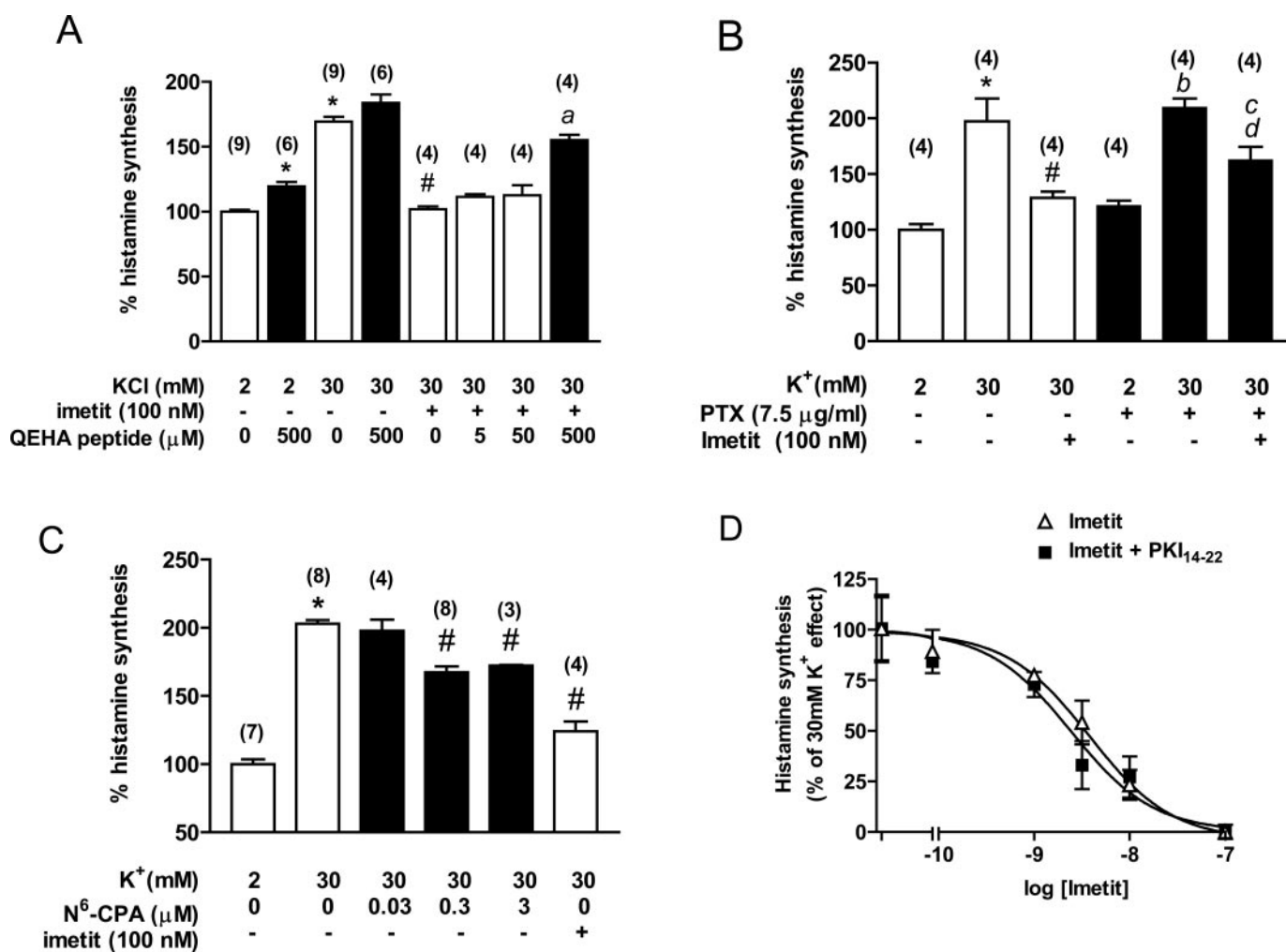


**Fig. 4.** H$_3$ receptor inhibits depolarization-stimulated histamine synthesis through actions of G$_{i/o}$ protein $\beta\gamma$ subunits, probably closing voltage-sensitive calcium channels A, Myr-QEHA peptide, a blocker of G protein $\beta\gamma$ subunits, impairs the effects of imetit by 79%. B, preincubation of slices with pertussis toxin (PTX) for 3 h reduces imetit effects by 31%. Mean 100% histamine synthesis in 2 mM K$^+$ controls after 3-h incubation was 6 fmol mg protein$^{-1}$ h$^{-1}$ (compared with 12–20 fmol mg protein$^{-1}$ h$^{-1}$ in the rest of experiments) probably caused by the short half-life of HDC. C, another G$_{i/o}$-coupled receptor, adenosine A$_1$, also inhibits depolarization-stimulated histamine synthesis. The maximal effect of the A$_1$ agonist $N^6$-cyclopentyladenosine (N$^6$-CPA) was a 33% inhibition. D, protein kinase A (PKA) blockade with 10 $\mu$M PKI$_{14-22}$ does not prevent imetit or depolarization effects. Myr-QEHA and PKI$_{14-22}$ were applied 15 min before depolarization. The agonists were added 10 min before depolarization. Results represent means $\pm$ S.E.M. Number of slice incubations are indicated in brackets above the columns. D, number of slice incubations is two to four per group, and no differences were found between curves. $\star$, $p < 0.05$ compared with 2 mM K$^+$; #, $p < 0.05$ compared with 30 mM K$^+$; $a$, $p < 0.05$ compared with 30 mM K$^+$ + imetit; $b$, $p < 0.05$ compared with 2 mM K$^+$ + PTX; $c$, $p < 0.05$ compared with 30 mM K$^+$ + PTX, one-way ANOVA plus Bonferroni post hoc tests; $d$, $p < 0.05$ compared with 30 mM K$^+$ + imetit, two-tailed Student's $t$ test.

whether this pathway would alter $H_3$ agonist inhibition of depolarization effects. The effects of the $H_3$ agonist imetit at different concentrations were not significantly potentiated by the presence of myristoylated PKA inhibitor peptide $PKI_{14-22}$ at 10 $\mu$M (Fig. 4D). This concentration of the PKA inhibitor is effective in blocking PKA stimulation of histamine synthesis, but it has no effects on depolarization (Fig. 5A) (Gomez-Ramirez et al., 2002). These results strengthen our view that there is no participation of the adenylate cyclase/PKA pathway on histamine synthesis activation by depolarization, and $H_3$ receptors do not seem to inhibit depolarization effects using the adenylate cyclase/PKA pathway.

Because calcium modulates certain isoforms of adenylate cyclases (Sunahara et al., 1996) and a stimulation the adenylate cyclase/PKA pathway activates histamine synthesis (Gomez-Ramirez et al., 2002), we were also interested in testing whether this effect could be related to a facilitation of calcium entry. As reported in Gomez-Ramirez et al. (2002), phosphodiesterase inhibition by IBMX (1 mM) stimulated histamine synthesis in the absence of depolarization. This effect was completely prevented by the PKA inhibitors $PKI_{14-22}$ (10 $\mu$M) (Fig. 5A) and Rp-adenosine-3'5'-cyclic monophosphate (500 $\mu$M) (Gomez-Ramirez et al., 2002), indicating that it is caused by PKA activation by an increase of cAMP levels. The effects of IBMX were also reverted by the histamine $H_3$ agonist imetit (Fig. 5C) (Gomez-Ramirez et al., 2002). In contrast, no inhibitor of calcium entry or CaMKII tested altered IBMX effects significantly (3 $\mu$M $\omega$-conotoxin MVIIC, 3 $\mu$M $\omega$-conotoxin GVIA, 1 mM W-13, and 30 $\mu$M KN-62) (Fig. 5A). Thus, the stimulations of histamine synthesis elicited by IBMX and depolarization should be mediated by separate mechanisms. To confirm such a hypothesis, we incubated the slices in the presence of either 1 mM IBMX, 30 mM KCl, or both. The stimulations elicited by each agent individually did not change by their concomitant administration, seeming to be purely additive (Fig. 5B). In addition, as expected, the effects of imetit on the stimulation elicited by IBMX did not depend on calcium entry through N-channels (Fig. 5C). These results suggest that histamine synthesis can be stimulated independently by the adenylate cyclase/PKA pathway or by calcium entry/CaMKII, and both pathways are inhibited by $H_3$ receptors separately.

If depolarization effects on histamine synthesis are mediated by CaMKII phosphorylation of HDC, IBMX effects could be mediated by PKA phosphorylation. In fact, consensus sites for PKA phosphorylation of HDC are known (Joseph et al., 1990) (http://www.cbs.dtu.dk/databases/PhosphoBase/predict/predform.html), although to our knowledge, phosphorylation has not been reported. Thus, we incubated purified recombinant 1/512 HDC in the presence of PKA and [$\gamma$-$^{32}$P]ATP. Autoradiography of vacuum-dried SDS-PAGE gels resulted in a clear $^{32}$P incorporation into the protein (Fig. 6A). Incubation controls were not different from background. Furthermore, activity of phosphorylated HDC in the presence of 60 $\mu$M histidine (0.1 $\mu$M [$^3$H]histidine) (Ortiz et al., 2000) was 22% higher than their respective nonphosphorylated controls (Fig. 6B). Thus, like CaMKII, PKA is also able to phosphorylate HDC, although the activation it elicits in vitro seems smaller than in the case of CaMKII.
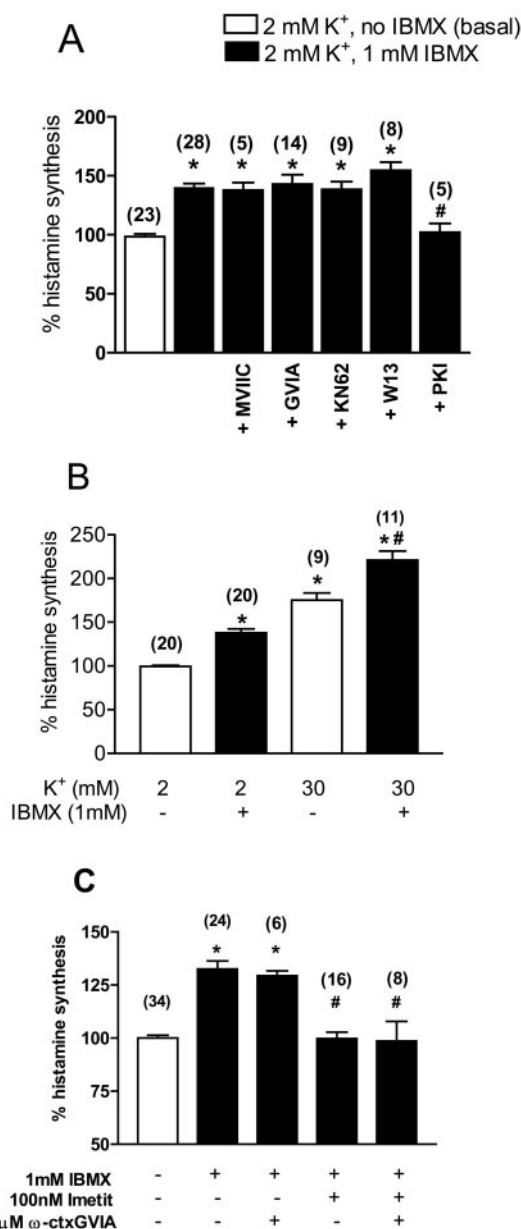
**Fig. 5.** Independent stimulation of histamine synthesis in brain cortical slices by potassium depolarization and by IBMX (a phosphodiesterase inhibitor elevating cAMP levels). A, the stimulation of histamine synthesis by IBMX is not impaired by blockade of N,P/Q calcium channels (3 $\mu$M $\omega$-conotoxin MVIIC), of N-calcium channels (3 $\mu$M $\omega$-conotoxin GVIA), by the calmodulin inhibitor W-13 (1 mM), or by the CaMKII inhibitor KN-62 (30 $\mu$M). In contrast, the PKA inhibitor $PKI_{14-22}$ (10 $\mu$M) suppressed IBMX effects. All inhibitors except IBMX were added to the incubation 20 min before [$^3$H]histidine. IBMX was added 15 min before [$^3$H]histidine. Results represent means $\pm$ S.E.M. Number of slice incubations are indicated in brackets above the columns. Mean 100% of histamine synthesis in 2 mM K$^+$ controls was 15 fmol mg protein$^{-1}$ h$^{-1}$. $\star$, $p < 0.01$ compared with controls (2 mM K$^+$); #, $p < 0.01$ compared with IBMX. B, IBMX effects are additive to those of depolarization. IBMX was added 15 min before [$^3$H]histidine, and thereafter samples were depolarized with 30 mM KCl for 30 min or kept in 2 mM KCl. Mean 100% of histamine synthesis was 18 fmol mg protein$^{-1}$ h$^{-1}$. $\star$, $p < 0.01$ compared with basal values; #, $P < 0.01$ compared with 30 mM K$^+$ or IBMX stimulation. C, IBMX effects are prevented by incubation with the $H_3$ agonist imetit, even in the presence of $\omega$-conotoxin GVIA. Imetit and GVIA were added 20 min before [$^3$H]histidine. IBMX was added 15 min before [$^3$H]histidine. Mean 100% 2 mM K$^+$ controls were 14 fmol mg protein$^{-1}$ h$^{-1}$. $\star$, $p < 0.01$ versus basal values; #, $p < 0.01$ versus IBMX.

## Discussion

This article is the first report of the transduction pathways used by $H_3$ autoreceptors to modulate histamine synthesis in nerve endings stimulated by depolarization. It was well established that neuronal histamine synthesis can be stimulated by depolarization with KCl (Arrang et al., 1987), although the biochemical mechanisms involved remained unknown. The requirement of extracellular calcium entry through voltage-sensitive channels was expected. In particular, the involvement of N-type channels was not surprising because they are abundant in nerve terminals and participate in the release of histamine (Takemura et al., 1989) and other neurotransmitters (Kerr and Yoshikami, 1984; Rittenhouse and Zigmond, 1999). However, additional calcium channel subtypes (perhaps P-channels) seem to be involved, because the blockade of depolarization effects by $\omega$-conotoxin GVIA is not complete (Fig. 1) (Takemura et al., 1989). Using voltage-clamp electrophysiology, Takeshita et al. (1998) found N- and P-calcium channels to mediate histamine $H_3$ receptor effects in histaminergic neurons. However, it also could be possible that different channel subtypes participate in the regulation of histamine synthesis in histaminergic cell bodies—located in the tuberomamillary nuclei—versus nerve endings because it occurs in catecholaminergic neurons (Rittenhouse and Zigmond, 1999).

Because CaMKII is highly enriched in brain and it participates in the stimulation by depolarization of tyrosine and



**Fig. 6.** Protein kinase A phosphorylates and activates recombinant 1/512 HDC in vitro. A, autoradiogram showing the incorporation of $^{32}$P into a protein of the expected molecular size of 1/512 HDC in the presence of PKA. B, PKA-phosphorylated samples have higher histidine decarboxylase activity. Activity was determined in the presence of 60 $\mu$M histidine and 0.1 $\mu$M [$^3$H]histidine after PKA phosphorylation in vitro. Results are means $\pm$ S.E.M. Number of determinations is indicated in brackets above the columns. Mean 100% of HDC activity was 1482 nmol mg protein$^{-1}$ h$^{-1}$. $\star$, $p < 0.001$ two-tailed Student's $t$ test.

tryptophan hydroxylases (Yamauchi et al., 1981; El Mestikawy et al., 1983; Itagaki et al., 1999), we tested the hypothesis that extracellular calcium entry would stimulate histamine synthesis through this enzyme. We used three structurally different inhibitors of CaMKII with different inhibitory mechanisms, given that some of them have nonspecific effects (see below). W-13 is a naphthalene-sulfonamide molecule that interacts with calcium/calmodulin and impairs its hydrophobic binding to targets (Tanaka et al., 1982). The isoquinoline molecule KN-62 interacts with CaMKII, inhibiting calcium/calmodulin binding competitively (Tokumitsu et al., 1990). In contrast, the myristoylated-AIP is a 13-mer peptide that competitively inhibits CaMKII autophosphoryation required for kinase activity (Ishida et al., 1995). All three CaMKII inhibitors shut down depolarization induction of histamine synthesis by a similar 91 to 93%, suggesting that their effect is probably caused by the impairment of CaMKII activity. Nonspecific effects reported for KN-62 and some calmodulin inhibitors include the impairment of $^{45}$C-calcium uptake into nerve terminals (Greenberg et al., 1987). However, in our case, nonspecific effects seem to be less relevant than CaMKII inhibition, because the myristoylated-AIP blocked depolarization effects to a similar extent. Although reported IC$_{50}$ values of CaMKII inhibition for the three compounds are much lower than the concentrations needed in slices, we note that diffusion across membranes is a principal factor determining the potency of intracellularly acting compounds. The biggest difference corresponded to AIP (IC$_{50}$ = 40 nM) (Ishida et al., 1995), a peptide whose hydrophilic sequence greatly inhibits membrane crossing. For this reason, in parallel experiments, we also attempted to determine the degree of phosphorylation of HDC in the slices by using immunoprecipitations, but in our hands, this technique did not allow for the acquisition of quantitative results. Our qualitative experiments (data not shown), however, suggest that depolarization increases HDC phosphorylation in slices, and AIP and KN-62 block most of this effect.

In line with the previous observation, we show that CaMKII can directly phosphorylate a recombinant version of the histamine-synthesizing enzyme (HDC) and increase its activity. The full-length HDC protein (74 kDa) contains six putative CaMKII phosphorylation sites, four of which (Thr$^{34}$, Ser$^{154}$, Ser$^{362}$, and Thr$^{385}$) are within the sequence of recombinant 1/512 HDC. Further work will examine which sites were phosphorylated under our conditions as well as the kinetic parameters modified by CaMKII phosphorylation. It is generally accepted that carboxyl-terminal truncation of the full-length protein yields a catalytically active form of $M_r$ 55,000, whose properties are matched closely by 1/512 HDC (Taguchi et al., 1984; Joseph et al., 1990; Engel et al., 1996; Olmo et al., 2002; Rodriguez-Caso et al., 2003), although controversy about the size of the functional forms of HDC in vivo still exists (Dartsch et al., 1999; Fleming and Wang, 2003). On the other hand, CaMKII activation of HDC does not seem to require additional proteins, because we can increase its activity in vitro using purified enzymes. In this sense, HDC seems to differ from tyrosine and tryptophan hydroxylases, which require the adaptor protein 14-3-3 to increase their catalytic activity after CaMKII phosphorylation (Yamauchi et al., 1981; Itagaki et al., 1999). In vivo,
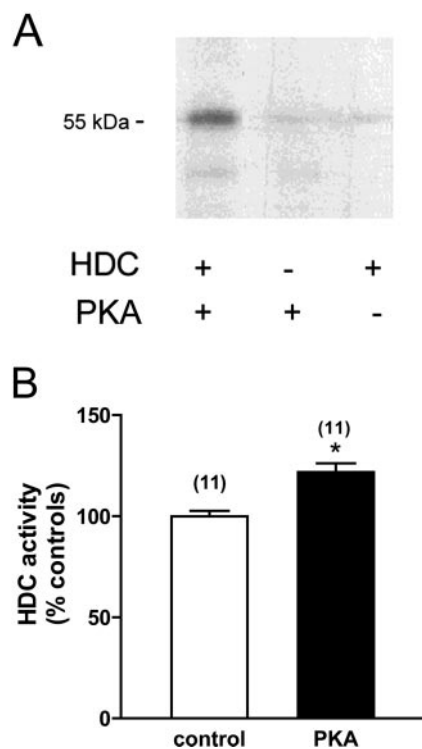
nevertheless, we cannot exclude the possibility that other proteins may modulate HDC activation by CaMKII.

Incubation of slices with an $H_3$ receptor agonist prevented the effects of depolarization on histamine synthesis (Arrang et al., 1987; Gomez-Ramirez et al., 2002) (Fig. 4). This result suggests that the histamine $H_3$ receptor at some point inhibits the pathway leading to CaMKII phosphorylation of HDC. This action is exerted probably on calcium entry through N- and P-type calcium channels, as reported by Takeshita et al. (1998). Calcium channels are inhibited by G protein $\beta\gamma$ subunits (Diverse-Pierluissi et al., 2000) that should be released by agonist binding to the $H_3$ receptor. To evaluate the participation of $\beta\gamma$ subunits in agonist effects, we applied a peptide acting as scavenger of these subunits to the slice incubation (QEHA peptide in myristoyl form) (Chen et al., 1995) (Fig. 4A). To the best of our knowledge, this is the first report of an action of the QEHA peptide applied extracellularly in myristoyl form. Myristoylation was performed to increase its lipophility and help it in crossing membranes. Our results demonstrate that the Myr-QEHA peptide impaired the actions of imetit, which suggests that it did cross membranes as expected and bound G protein $\beta\gamma$ subunits, impairing their action on calcium channels. The concentrations at which the peptide blocked imetit actions were only two to five times higher than those blocking $\beta\gamma$ effects on adenylate cyclase 2 in disrupted membrane preparations (Chen et al., 1995). It should be noted that other myristoylated peptides used in this article against intracellular targets ($PKI_{14-22}$, AIP) also required higher concentrations when applied to slices of tissue than those reported in homogenate assays. On the other hand, inhibition of calcium currents by $H_3$ receptors in histaminergic neurons seems to be quite small (Takeshita et al., 1998; Brown et al., 2001), whereas $H_3$ receptors elicit a near-complete inhibition of stimulated histamine synthesis in our preparations. Because $H_3$ receptors do not seem to be coupled to potassium channels that could amplify calcium entry (Brown et al., 2001), we further explored the mechanisms involved. First, we tested whether pertussis toxin ADP-ribosylation of $G_{i/o}$ proteins would impair imetit effects. Even though the short half-life of HDC decreases histamine synthesis too rapidly compared with the rate of pertussis toxin internalization, the toxin reduced the effects of imetit. Next, we sought other $G_{i/o}$-coupled receptors that would reduce depolarization effects on histamine synthesis. It was not surprising that adenosine $A_1$ receptors did, because Brown and Haas (1999) reported that $A_1$ and $H_3$ receptors elicit calcium-channel inhibition on hippocampal glutamatergic terminals. We found that maximal effects of the $A_1$ agonist were smaller than those of the $H_3$ agonist, which suggests that $A_1$ receptors recruit part of the transduction machinery of $H_3$ receptors. On the other hand, the effector enzyme adenylate cyclase can be inhibited by histamine $H_3$ receptors (Lovenberg et al., 1999; Gomez-Ramirez et al., 2002), modulated by $\beta\gamma$ subunits (Tang and Gilman, 1991), and downstream PKA can phosphorylate and activate some types of calcium channels (Rotman et al., 1995; Tamse et al., 2003). Thus, we tested whether additional actions of $H_3$ receptors through adenylate cyclases might potentiate the inhibition of calcium channels. However, such actions would not easily fit with our previous finding that PKA inhibition does not modify the stimulation of histamine synthesis by depolarization (Gomez-Ramirez et al., 2002)

(Fig. 4D). We show that PKA blockade did not significantly modify the inhibition curve of the $H_3$ agonist at different concentrations (Fig. 4D), confirming that the adenylate cyclase/PKA pathway does not seem to modulate depolarization effects. This result supports the interpretation that a direct inhibition of N-type calcium channels by G protein $\beta\gamma$ subunits accounts for $H_3$ receptor repression of depolarization, impairing CaMKII and HDC activation. Synaptic mechanisms might amplify the magnitude of the effects of $H_3$ receptors on histamine synthesis, for instance, if $H_3$ receptors were clustered with N-channels, CaMKII, and HDC.

Although PKA inhibition does not impair depolarization effects, a stimulation of PKA by inhibition of cAMP breakdown with IBMX activates histamine synthesis (Gomez-Ramirez et al., 2002) (Fig. 5A). Thus, we wanted to test whether IBMX effects could be attributable to phosphorylation by PKA of calcium channels and facilitation of calcium entry. We found no evidence supporting this, because calcium-entry blockers and CaMKII inhibitors did not modify IBMX effects (Fig. 5A). Moreover, the stimulations of histamine synthesis elicited by IBMX and depolarization were apparently additive and mediated by independent mechanisms (Fig. 5B). A similar result was found previously for tyrosine hydroxylase, an enzyme independently activated by cAMP- or calcium-dependent phosphorylation (El Mestikawy et al., 1983). We also found that PKA phosphorylates and activates HDC in vitro (Fig. 6), suggesting that the same occurs in slices after incubation with IBMX. It is noteworthy that both in vitro and in slices, PKA effects are smaller than CaMKII effects. HDC phosphorylation seems to be the biochemical endpoint of both pathways modulating histamine synthesis. In fact, the similarity between the mechanisms activating different neurotransmitter synthesis enzymes is striking. Different types of neurons seem to determine how much neurotransmitter must be synthesized, depending on similar mechanisms. Neurotransmitter synthesis enzymes may be a biochemical point of integration of the diverse signals that neurons receive.

In conclusion, our results show that calcium entry during depolarization activates histamine synthesis through CaMKII phosphorylation. $H_3$ autoreceptors in brain nerve terminals inhibit both calcium entry and adenylate cyclase pathways, repressing both mechanisms that could activate histamine synthesis.

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