

**H<sub>3</sub> AUTORECEPTORS MODULATE HISTAMINE SYNTHESIS  
THROUGH CALCIUM/CALMODULIN- AND cAMP-DEPENDENT  
PROTEIN KINASE PATHWAYS**

Anna Torrent, PhD, David Moreno-Delgado, BS, Jordi Gómez-Ramírez, PhD,  
Daniel Rodríguez-Agudo, PhD, Carlos Rodríguez-Caso, BS, Francisca  
Sánchez-Jiménez, PhD, Isaac Blanco, PhD and Jordi Ortiz, PhD

Universitat Autònoma de Barcelona, Neuroscience Institute,  
Dept. Biochemistry & Molecular Biology  
Bellaterra (Spain)

Running title: Modulation of histamine synthesis by H<sub>3</sub> receptors

Corresponding author:

Jordi Ortiz, PhD

Universitat Autònoma de Barcelona

Dept. Biochemistry & Molecular Biology

School of Medicine, Neuroscience Institute, Room M2-120

E-08193 Bellaterra (Spain)

Email: Jordi.Ortiz@uab.es

Phone +34 677 392 828 (cellular) / +34 935 811 625

Fax: +34 935 811 573

27 text pages

0 tables

6 figures

191 words in Abstract

465 words in Introduction

1633 words in Discussion

Abbreviations: AIP = Myristoylated autocamtide-2-related inhibitory peptide; CaMKII = Calcium/calmodulin-dependent protein kinase type II; IBMX = 1-Methyl-3-isobutylxanthine; HDC = Histidine decarboxylase; KN-62 = 1-[N,O-bis(5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine; PBS = Phosphate-buffered saline; PKA = Protein kinase A; PKI<sub>14-22</sub> = Myristoylated protein kinase A inhibitor amide 14-22; PTX = pertussis toxin; Myr-QEHA peptide = Myristoyl-N-QEHAQEPERQYMHIGTMVEFAYALVGK; W-13 = N-(4-aminobutyl)-5-chloro-2-naphthalenesulfonamide.

ABSTRACT:

H<sub>3</sub> 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<sub>3</sub> agonists. We show that histamine synthesis stimulation by depolarization with 30 mM K<sup>+</sup> 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<sub>3</sub> receptor agonist imetit was impaired by preincubation with pertussis toxin as well as by the presence of a myristoylated peptide (Myr-QEHA peptide) blocking the actions of G-protein betagamma subunits. The stimulation of another G<sub>i/o</sub>-coupled receptor, adenosine A<sub>1</sub>, also decreased depolarization-stimulated histamine synthesis. In contrast, protein kinase A activation -which is also repressed by H<sub>3</sub> 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<sub>3</sub> autoreceptors.

Histaminergic neurons modulate several physiological processes, such as sleep/wake cycles, food intake and memory (see review by Brown et al., 2001). Four subtypes of histamine receptors (named H<sub>1</sub> to H<sub>4</sub>) have been identified and cloned. The H<sub>3</sub> is the only subtype present in histaminergic neurons where it mediates inhibition of histamine release and synthesis, functioning as a classical autoreceptor (Arrang et al., 1983, 1987). H<sub>3</sub> receptors are also expressed by other neurons, mostly cortical, thalamic and striatal projection neurons (Pillot et al., 2002). Structurally, H<sub>3</sub> receptors are members of the seven transmembrane receptor superfamily (Lovenberg et al., 1999) and couple to G<sub>i/o</sub> proteins (Clark and Hill, 1996). Stimulation of H<sub>3</sub> 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 MAP kinase phosphorylation in receptor-transfected cells (Drutel et al., 2001). It has been shown that H<sub>3</sub> autoreceptors have significant constitutive activity, resulting into partial tonic autoinhibition of histaminergic neurons in the absence of histamine (Morisset et al., 2000).

In spite of the potential utility and the many pharmacological effects described for H<sub>3</sub> receptor ligands (Brown et al., 2001), little is known about the transduction mechanisms triggered by H<sub>3</sub> receptors within histaminergic neurons. These neurons are characterized by the presence of the enzyme synthesizing histamine, histidine decarboxylase (HDC). HDC is a pyridoxal-5'-phosphate dependent decarboxylase translated as a 74 kDa precursor which undergoes carboxy-terminal truncation in order 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<sub>3</sub> receptors (Gomez-Ramirez et al., 2002). As protein kinase A (PKA) activation was required also for the effects of H<sub>3</sub> receptor inverse agonists, we concluded that this pathway mediated H<sub>3</sub> autoreceptor inhibition of histamine synthesis. However, we also showed that histamine synthesis can be stimulated by depolarization with K<sup>+</sup> ions independently of PKA blockade (Gomez-Ramirez et al., 2002). Because H<sub>3</sub> agonists inhibit the effects of depolarization (Arrang et al., 1987), we hypothesize that H<sub>3</sub> receptors must be coupled to another transduction pathway modulating histamine synthesis besides adenylate cyclase /

PKA. Our goal in the present work was to investigate such 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. Accordingly, a recombinant version of catalytically active HDC can be phosphorylated *in vitro* by both PKA and CaMKII, leading to increased activity. In brain slices, stimulation of H<sub>3</sub> receptors represses the effects elicited through either of these kinases, which apparently are mutually independent.

## MATERIALS AND METHODS

*Chemicals:* Ring-labelled [2,5-<sup>3</sup>H]L-histidine (50 Ci / mmol) obtained from Amersham (Buckinghamshire, UK) was purified by high performance liquid chromatography (HPLC) before use as described in Ortiz et al. (2000). PKI<sub>14-22</sub>, IBMX, KN-62, W-13, AIP, pertussis toxin, calmodulin and CaMKII were obtained from Calbiochem/Merck KGaA (Darmstadt, Germany). Omega-conotoxins MVIIC and GVIA were purchased from Tocris Cookson (Bristol, UK). The betagamma inhibitor 27-mer QEHA peptide (Chen et al., 1995) was synthesized and N-terminal myristoylated by Pepscan (Netherlands). Imetit, HPLC standards and other reagents were purchased from Sigma/RBI (Steinheim, Germany). OptiPhase HiSafe-3 liquid scintillation cocktail was from Perkin Elmer Wallac (Turku, Finland).

*Preparation and incubation of brain slices:* Male Sprague-Dawley rats of 200 to 250 g (Animal Service, Universitat Autònoma de Barcelona) were killed by decapitation between 10-11 h 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<sub>2</sub>, 0.67 mM MgSO<sub>4</sub>, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, 27.5 mM NaHCO<sub>3</sub>, 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 x 0.3 mm side. The miniprisms were suspended in ice-cold Krebs-Ringer-bicarbonate medium and washed to remove debris of damaged cells. 100 µl aliquots 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 a Eppendorf Thermomixer under O<sub>2</sub>/CO<sub>2</sub> (95:5) atmosphere. Previously purified [<sup>3</sup>H]-histidine was added to all samples (6.25 µCi per tube, to make a final concentration of 0.5 µM) and the tubes were incubated for 5 minutes to allow [<sup>3</sup>H]-histidine uptake. If depolarizing conditions were required, buffer was added with concentrated KCl upto a total volume of 250 µl. NaCl concentration in depolarizing buffer was decreased proportionally to maintain isotonicity. Miniprisms were then incubated for 30 minutes to synthesize [<sup>3</sup>H]-histamine. When drugs were tested (H<sub>3</sub> ligands, phosphodiesterase or kinase inhibitors, calcium channel-blockers) they were added 15 or 20 min before depolarization. Several drug concentrations were routinely tested, since diffusion across the slices may be very different between compounds. This is specially important for protein kinase inhibitors, as relatively high concentrations were needed to achieve intracellular effects (see Figure 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 trichloroacetic acid before [<sup>3</sup>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. 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 x g, 10 min, 4 °C) and supernatants recovered and processed as follows in [<sup>3</sup>H]-Histamine Purification by HPLC.

*[<sup>3</sup>H]-Histamine purification by HPLC:* Deproteinized supernatants were mixed with 100 µl ion-exchange resin (Amberlite IRA 900 mesh 16-50, Supelco, prepared as described in 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 % [<sup>3</sup>H]-histidine, increasing the sensitivity of subsequent [<sup>3</sup>H]-histamine purification. The supernatants were injected into a Merck-Hitachi HPLC system equipped with a L-7200 autosampler and a reverse-phase C18 column (25 x 0.46 cm Tracer Extrasil ODS-2 5-µm particle size, obtained from Teknokroma, Spain) with 2 x 20 mm guard column (Upchurch). 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 - 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 [<sup>3</sup>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 dpm counted. Recovery (typically about 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, France). [<sup>3</sup>H]-histamine synthesis in each sample was estimated from dpm obtained corrected by recovery, blank dpm and specific activity of [<sup>3</sup>H]-histidine, and 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-PCR 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, USA) as described in Olmo et al (2002). The vector was expressed in BL21(DE3)pLysS competent E. coli cells by induction with isopropyl thiogalactoside following manufacturer's instructions. The cells were pelleted by centrifugation, resuspended in 20 mM potassium buffer pH 7.0 containing 2 mM EDTA, 5 mM pyridoxal-5'-phosphate and 1 % polyethylene glycol 300, and lysed by sonication. The lysate was centrifuged for 90 min at 14,000 rpm, 4 °C and the supernatant was recovered for purification of HDC protein. Purification was performed following the procedure described in Olmo et al. (2002). All purification steps were monitored by SDS-PAGE and western blot using a 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, Pharmacia Biotech, Sweden) and eluted with buffer containing 1 mM potassium phosphate pH 7.0, 0.1 mM dithiothreitol, 5 μM pyridoxal-5'-phosphate and 1% polyethylene glycol 300. The eluates were concentrated by ultrafiltration in Amicon cells and subjected to DEAE interchange chromatography (DEAE-Toyopearl column 650M, Tosoh, Japan) using a linear NaCl gradient in the

previous buffer. HDC eluted at 0.1 M NaCl. Finally, hydroxyapatite chromatography (HTP, BioRad) was used in a potassium phosphate gradient raising up to 200 mM phosphate. Both by western blot and Coomassie blue staining the purified fraction yielded a single band at Mr 55,000, the expected size of 1/512 HDC.

*Phosphorylation and activity of recombinant HDC:* For CaMKII phosphorylation, 30 µg of purified HDC were incubated in 50 mM HEPES buffer pH 7.4 in the presence of 60 ng CaMKII (Calbiochem), 3 µg calmodulin (Calbiochem), 0.3 mM CaCl<sub>2</sub>, 5 mM MgCl<sub>2</sub> and 50 µM ATP (containing 10 µCi gamma-<sup>32</sup>P-ATP; Amersham) in a total volume of 100 µl. Incubations were carried out for 40 min at 30 °C and then stopped by addition of SDS-PAGE sample buffer. Control samples were incubated without CaMKII. For PKA phosphorylation, the same conditions were used except that 10 µg of purified HDC were incubated with 0.1 µg PKA (catalytic subunit from bovine heart, Sigma). Calmodulin and CaCl<sub>2</sub> were omitted. Samples were boiled for 5 min and 10 µl were subjected to SDS-PAGE in gels containing 10 % polyacrylamide. Molecular weight markers used were BioRad Precision Plus<sup>TM</sup>. 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. Radiolabelled bands were quantified by densitometry using Scion Image software (Scioncorp, USA). To determine changes in HDC activity, 0.45 µg of HDC were phosphorylated in the above conditions except that gamma-<sup>32</sup>P-ATP was omitted. Thereafter, phosphorylated HDC and control samples were incubated in 50 mM HEPES buffer pH 7.4, 5 mM MgCl<sub>2</sub>, 10 µM pyridoxal-5'-phosphate and 60 µM histidine (0.1 µM [<sup>3</sup>H]-His of 43 Ci/mmol) in a final volume of 100 µl. Samples were incubated for 30 min at 37 °C and [<sup>3</sup>H]-histamine formation was quantified as described in Ortiz et al (2000).

*Statistical analysis:* As 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 was normalized to % 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 non-selective 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. 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 (Figure 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 & Yoshikami, 1984) blunted depolarization effects by 84 % (Figure 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 appears to contribute to a minor extent.

Next we looked for 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; Figure 2A). Depolarization effects were almost completely suppressed (-92%) at the highest concentration of W-13 assayed (1 mM). This dose of W-

13 may appear relatively high as compared to the IC<sub>50</sub> of calmodulin inhibition in homogenates (about 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 in order to reach its intracellular target. Subsequently, we tested two different CaMKII inhibitors: KN-62 (Tokumitsu et al., 1990) and the myristoylated autocalmitide-2-related inhibitory peptide (AIP; Ishida et al., 1995). Both agents prevented histamine synthesis stimulation by depolarization in a dose-dependent manner (Figure 2, B and C). Maximal inhibition of depolarization effects was 93 and 91 % for KN-62 and the inhibitor peptide, respectively. As expected, all these compounds needed at least one order of magnitude higher concentrations when used in slices as compared to their reported IC<sub>50</sub> in homogenates, a difference that was even greater in the case of AIP whose highly hydrophilic sequence should difficult membrane crossing.

The previous results raised the question of whether CaMKII could directly phosphorylate the histamine synthesizing enzyme, HDC. Several potential phosphorylation sites could be predicted on the basis of 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 carboxy-terminal truncation in order to be catalytically active (Joseph et al., 1990). We obtained purified HDC by bacterial expression of a plasmid encoding 512 aminoacids of the N-terminal side of the rat enzyme (1/512 HDC), which yields a catalytically active protein with similar size and characteristics than 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<sup>2+</sup>, calmodulin and gamma-<sup>32</sup>P-ATP resulted in strong <sup>32</sup>P incorporation, as determined by autoradiography of vacuum-dried SDS-PAGE gels. <sup>32</sup>P labelled a protein of Mr 55,000, the expected size of 1/512 HDC (Figure 3A, right lane). Incubation controls containing CaMKII, Ca<sup>2+</sup>, calmodulin and gamma-<sup>32</sup>P-ATP but lacking purified HDC showed a minor incorporation of <sup>32</sup>P into proteins of similar molecular weight (Figure 3A, left line), which is likely due to the expected autophosphorylation of CaMKII alpha and beta subunits of 50 - 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 (Figure 3A, middle lane).

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

The most likely mechanism by which  $\text{H}_3$  receptors could repress histamine synthesis activation by depolarization would be the inhibition of voltage-sensitive calcium channels by G protein betagamma subunits (Takeshita et al., 1998; Diverse-Pierluissi et al., 2000). To test whether blockade of betagamma actions would prevent the effects of a histamine  $\text{H}_3$  agonist, we incubated brain slices in the presence of different concentrations of a peptide (QEHA peptide) which binds to betagamma subunits, impairing their interaction with targets (Chen et al., 1995). The QEHA peptide has been shown previously to inhibit the actions of G protein betagamma subunits on several effectors such as adenylate cyclases, G protein-regulated kinase and a  $\text{K}^+$  channel (Chen et al., 1995). The peptide we used was N-terminal myristoylated in order 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 % (Figure 4A). The peptide alone elicited a 19 % increase on basal histamine synthesis (Figure 4A). To confirm that  $\text{G}_{i/o}$  protein subunits were involved in the effects of imetit, we incubated slices in the presence of 7.5  $\mu\text{g/ml}$  pertussis toxin for 3 h. The use of pertussis toxin in tissue slices has the disadvantage that it requires long incubation times in order to be internalized. However, in slices incubated for 3 h histamine synthesis was 63 % lower, probably due to the short half-life of histidine decarboxylase (Engel et al., 1996; Rodriguez-Caso et al., 2003). Nevertheless, as 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 %

(Figure 4B). To further analyze the G proteins participating 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  $5-HT_{1B}$  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. Betagamma subunits should inhibit calcium channels (Takeshita et al., 1998; Diverse-Pierluissi et al., 2000) although additional actions on adenylate cyclases could not be discarded. Since histamine  $H_3$  receptors can modulate also 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 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 (Figure 4D). This concentration of the PKA inhibitor is effective in blocking PKA stimulation of histamine synthesis, but it has no effects on depolarization (see Figure 5A and Gomez-Ramirez et al., 2002). These results strength 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 appear 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 the this effect could be related to a facilitation of calcium entry. As reported in Gomez-Ramirez et al. (2002), phosphodiesterase inhibition by 1-methyl-3-isobutylxanthine (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; Figure 5A) and Rp-adenosine-3'5'-cyclic monophosphate (500  $\mu$ M; Gomez-Ramirez et al., 2002), indicating that it is due to PKA activation by increased of cAMP levels. The effects of IBMX were also reverted by the histamine  $H_3$  agonist imetit (Figure 5C and Gomez-Ramirez et al., 2002). In contrast, no

inhibitor of calcium entry or CaMKII tested altered significantly IBMX effects (3  $\mu$ M omega-conotoxin MVIIC, 3  $\mu$ M omega-conotoxin GVIA, 1 mM W-13 and 30  $\mu$ M KN-62; Figure 5A). Thus, the stimulations of histamine synthesis elicited by IBMX and depolarization should be mediated by separate mechanisms. To confirm such 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, appearing to be purely additive (Figure 5B). In addition, as expected the effects of imetit on the stimulation elicited by IBMX did not depend on calcium entry through N-channels (Figure 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<sub>3</sub> 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 and <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-<sup>32</sup>P-ATP. Autoradiography of vacuum-dried SDS-PAGE gels resulted in a clear <sup>32</sup>P incorporation into the protein (Figure 6A). Incubation controls were not different from background. Furthermore, activity of phosphorylated HDC in the presence of 60  $\mu$ M His (0.1  $\mu$ M [<sup>3</sup>H]-His; Ortiz et al., 2000) was 22 % higher than their respective non-phosphorylated controls (Figure 6B). Thus, as CaMKII, PKA is also able to phosphorylate HDC, although the activation it elicits *in vitro* appears smaller than in the case of CaMKII.

## DISCUSSION

This paper is the first report of the transduction pathways used by H<sub>3</sub> 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 since they are abundant in nerve terminals and participate in the release of histamine (Takemura et al., 1989) and other neurotransmitters (Kerr & Yoshikami, 1984 ; Rittenhouse and Zigmond, 1999). However, additional calcium channel subtypes (perhaps P-channels) appear to be involved, as the blockade of depolarization effects by omega-conotoxin GVIA is not complete (Figure 1 and Takemura et al., 1989). Using voltage-clamp electrophysiology, Takeshita et al. (1998) found N- and P-calcium channels to mediate histamine H<sub>3</sub> receptor effects in histaminergic neurons. However, it could also be possible that different channel subtypes participate in the regulation of histamine synthesis in histaminergic cell bodies - located in the tuberomammillary nuclei - vs. nerve endings, as it occurs in catecholaminergic neurons (Rittenhouse and Zigmond, 1999).

Since CaMKII is highly enriched in brain and it participates in the stimulation by depolarization of tyrosine and 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 naphthalenesulfonamide 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 autophosphorylation required for kinase activity (Ishida et al., 1995). All three CaMKII inhibitors shut down depolarization induction of histamine synthesis by a similar 91-93 %

suggesting that their effect is likely due to the impairment of CaMKII activity. Nonspecific effects reported for KN-62 and some calmodulin inhibitors include the impairment of [<sup>45</sup>C]-calcium uptake into nerve terminals (Greenberg et al., 1987). However, in our case nonspecific effects appear to be less relevant than CaMKII inhibition, as the myristoylated-AIP blocked depolarization effects to a similar extent. Although reported IC<sub>50</sub> of CaMKII inhibition for the three compounds are much lower than the concentrations needed in slices, we must note that diffusion across membranes is a principal factor determining potency of intracellularly acting compounds. The biggest difference corresponded to AIP (IC<sub>50</sub> 40 nM, Ishida et al. 1995), a peptide whose hydrophilic sequence greatly difficults 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 to obtain quantitative results. Our qualitative experiments (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<sup>34</sup>, Ser<sup>154</sup>, Ser<sup>362</sup> and Thr<sup>385</sup>) are within the sequence of recombinant 1/512 HDC. Further work will examine which sites were phosphorylated in our conditions as well as the kinetic parameters modified by CaMKII phosphorylation. It is generally accepted that carboxy-terminal truncation of the full length protein yields a catalytically active form of Mr 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, since we can increase its activity in vitro using purified enzymes. In this sense HDC appears to differ from tyrosine and tryptophan hydroxylases, which require the adaptor protein 14-3-3 in order to increase their catalytic activity after CaMKII phosphorylation (Yamauchi et al., 1981; Itagaki et al.,

1999). In vivo, nevertheless, we can not exclude the possibility that other proteins may modulate HDC activation by CaMKII.

Incubation of slices with a H<sub>3</sub> receptor agonist prevented the effects of depolarization on histamine synthesis (Arrang et al., 1987; Gomez-Ramirez et al., 2002; Figure 4). This result suggests that the histamine H<sub>3</sub> receptor inhibits at some point the pathway leading to CaMKII phosphorylation of HDC. This action is exerted likely on calcium entry through N- and P-type calcium channels, as reported by Takeshita et al. (1998). Calcium channels are inhibited by G protein betagamma subunits (Diverse-Pierluissi et al., 2000) that should be released by agonist binding to the H<sub>3</sub> receptor. To evaluate the participation of betagamma subunits in agonist effects, we applied to the slice incubation a peptide acting as scavenger of these subunits (QEHA peptide in myristoyl form, Chen et al., 1995; Figure 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 lipophilicity 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 betagamma subunits, impairing their action on calcium channels. The concentrations at which the peptide blocked imetit actions were only 2 to 5 times higher than those blocking betagamma 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<sub>14-22</sub>, 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<sub>3</sub> receptors in histaminergic neurons seems to be quite small (Takeshita et al., 1998; Brown et al., 2001), while H<sub>3</sub> receptors elicit a near-complete inhibition of stimulated histamine synthesis in our preparations. As H<sub>3</sub> receptors do not appear 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<sub>i/o</sub> proteins would impair imetit effects. Even though the short half-life of HDC decreases histamine synthesis too rapidly as compared to the rate of pertussis toxin internalization, the toxin reduced the effects of imetit. Next, we sought other G<sub>i/o</sub>-coupled receptors that would reduce depolarization effects on histamine



synthesis. It was not surprising that adenosine A<sub>1</sub> receptors did, as Brown and Haas (1999) reported that A<sub>1</sub>- and H<sub>3</sub>-receptors elicit calcium channel inhibition on hippocampal glutamatergic terminals. We found that maximal effects of the A<sub>1</sub> agonist were smaller than those of the H<sub>3</sub> agonist, which suggests that A<sub>1</sub> receptors recruit part of the transduction machinery of H<sub>3</sub> receptors. On the other hand, the effector enzyme adenylyl cyclase can be inhibited by histamine H<sub>3</sub> receptors (Lovenberg et al., 1999; Gomez-Ramirez et al., 2002), modulated by betagamma 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 if additional actions of H<sub>3</sub> receptors through adenylyl 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; Figure 4D). We show that PKA blockade did not significantly modify the inhibition curve of the H<sub>3</sub> agonist at different concentrations (Figure 4D), confirming that the adenylyl 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 betagamma subunits accounts for H<sub>3</sub> receptor repression of depolarization, impairing CaMKII and HDC activation. Synaptic mechanisms might amplify the magnitude of the effects of H<sub>3</sub> receptors on histamine synthesis, for instance -hypothetically- if H<sub>3</sub> 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 and Figure 5A). Thus, we wanted to test whether IBMX effects could be due to phosphorylation by PKA of calcium channels and facilitation of calcium entry. We found no evidence supporting this, as calcium entry blockers and CaMKII inhibitors did not modify IBMX effects (Figure 5A). Moreover, the stimulations of histamine synthesis elicited by IBMX and depolarization were apparently additive and mediated by independent mechanisms (Figure 5B). A similar result was previously found 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* (Figure 6), suggesting that the same occurs in slices after incubation with IBMX. Noteworthy, both *in vitro* and in slices PKA effects are smaller than CaMKII effects. HDC phosphorylation appears to be the biochemical endpoint of both pathways modulating histamine synthesis. In fact, the similarity on the mechanisms activating different neurotransmitter synthesis enzymes is striking. Different types of neurons appear to take decisions on 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<sub>3</sub> autoreceptors in brain nerve terminals inhibit both calcium entry and adenylate cyclase pathways, repressing both mechanisms that could activate histamine synthesis.

## REFERENCES

Arrang JM, Garbarg M, Schwartz JC (1983) Auto-inhibition of brain histamine release mediated by a novel class (H<sub>3</sub>) of histamine receptor. *Nature* **302**: 832-7.

Arrang JM, Garbarg M, Schwartz JC (1987) Autoinhibition of histamine synthesis mediated by presynaptic H<sub>3</sub>-receptors. *Neuroscience* **23**: 149-57.

Blandizzi C, Colucci R, Tognetti M, De Paolis B, Del Tacca M (2001) H<sub>3</sub> receptor-mediated inhibition of intestinal acetylcholine release: pharmacological characterization of signal transduction pathways. *Naunyn Schmiedebergs Arch Pharmacol* **363**: 193-202.

Brown RE & Haas, HL (1999) On the mechanism of histaminergic inhibition of glutamate release in the rat dentate gyrus. *J Physiol.* 515:777-86.

Brown RE, Stevens DR, Haas HL (2001) The physiology of brain histamine. *Prog Neurobiol.* **63**: 637-72.

Chafouleas JG, Bolton WE, Hidaka H, Boyd AE 3rd, Means AR. (1982) Calmodulin and the cell cycle: involvement in regulation of cell-cycle progression. *Cell* **28**: 41-50.

Chen J, DeVivo M, Dingus J, Harry A, Li J, Sui J, Carty DJ, Blank JL, Exton JH, Stoffel RH, Inglese J, Lefkowitz RJ, Logothetis DE, Hildebrandt JD, Iyengar R (1995) A region of adenylyl cyclase 2 critical for regulation by G protein beta gamma subunits. *Science* **268**: 1166-9.

Clark EA, Hill SJ. (1996) Sensitivity of histamine H<sub>3</sub> receptor agonist-stimulated [35S]GTP gamma[S] binding to pertussis toxin. *Eur J Pharmacol* **296**: 223-5.

Dartsch C, Persson L (1998) Recombinant expression of rat histidine decarboxylase: generation of antibodies useful for Western blot analysis *Int J Biochem Cell Biol.* **30**: 773-82.

Dartsch C, Chen D, Hakanson R, Persson L (1999) Histidine decarboxylase in rat stomach ECL cells: relationship between enzyme activity and different molecular forms. *Regulatory Peptides* **81**: 41-48.

Diverse-Pierluissi M, McInteire WE, Myung CS, Lindorfer MA, Garrison JC, Goy MF and Dunlap K (2000) Selective coupling of G-protein betagamma complexes to inhibition of Ca<sup>2+</sup> channels. *J Biol Chem* **275**: 28380-28385.

Drutel G, Peitsaro N, Karlstedt K, Wieland K, Smit MJ, Timmerman H, Panula P, Leurs R (2001) Identification of rat H<sub>3</sub> receptor isoforms with different brain expression and signaling properties. *Mol Pharmacol* **59**: 1-8.

El Mestikawy S, Glowinski J, Hamon M (1983) Tyrosine hydroxylase activation in depolarized dopaminergic terminals--involvement of Ca<sup>2+</sup>-dependent phosphorylation. *Nature* **302**: 830-2.

Engel N, Olmo MT, Coleman CS, Medina MA, Pegg AE, Sanchez-Jimenez F. (1996) Experimental evidence for structure-activity features in common between mammalian histidine decarboxylase and ornithine decarboxylase. *Biochem J.* **320**: 365-8.

Fleming JV, Wang TC. (2003) The production of 53-55-kDa isoforms is not required for rat L-histidine decarboxylase activity. *J Biol Chem.* **278**: 686-94.

Gomez-Ramirez J, Ortiz J, Blanco I (2002) Presynaptic H<sub>3</sub> autoreceptors modulate histamine synthesis through cAMP pathway. *Mol. Pharmacol.* **61**: 239-245.

Greenberg DA, Carpenter CL, Messing RO (1987) Interaction of calmodulin inhibitors and protein kinase C inhibitors with voltage-dependent calcium channels. *Brain Res* **404**: 401-4

Hillyard DR, Monje VD, Mintz IM, Bean BP, Nadasdi L, Ramachandran J, Miljanich G, Azimi-Zoonooz A, McIntosh JM, Cruz LJ, Imperial, JS, Olivera, BM (1992) A new Conus peptide ligand for mammalian presynaptic Ca<sup>2+</sup> channels. *Neuron* **9**: 69-77 .

Itagaki C, Isobe T, Taoka M, Natsume T, Nomura N, Horigome T, Omata S, Ichinose H, Nagatsu T, Greene LA, Ichimura T. (1999) Stimulus-coupled interaction of tyrosine hydroxylase with 14-3-3 proteins. *Biochemistry* **38**: 15673-80.

Ishida A, Kameshita I, Okuno S, Kitani T, Fujisawa H. (1995) A novel highly specific and potent inhibitor of calmodulin-dependent protein kinase II. *Biochem Biophys Res Commun.* **212**: 806-12.

Joseph DR, Sullivan PM, Wang YM, Kozak C, Fenstermacher DA, Behrendsen ME, Zahnaw CA (1990) Characterization and expression of the complementary DNA encoding rat histidine decarboxylase. *Proc Natl Acad Sci U S A.* **87**: 733-7.

Kerr LM, Yoshikami D (1984) A venom peptide with a novel presynaptic blocking action. *Nature* **308**: 282-4.

Lai Y, Nairn AC, Gorelick F, Greengard P. (1987) Ca<sup>2+</sup>/calmodulin-dependent protein kinase II: identification of autophosphorylation sites responsible for generation of Ca<sup>2+</sup>/calmodulin-independence. *Proc Natl Acad Sci U S A.* **84**: 5710-4.

Lovenberg TW, Roland BL, Wilson SJ, Jiang X, Pyati J, Huvar A, Jackson MR, Erlander MG. (1999) Cloning and functional expression of the human histamine H<sub>3</sub> receptor. *Mol Pharmacol* **55**: 1101-7.

Morisset S, Rouleau A, Ligneau X, Gbahou F, Tardivel-Lacombe J, Stark H, Schunack W, Ganellin CR, Schwartz JC, Arrang JM (2000) High constitutive activity of native H<sub>3</sub> receptors regulates histamine neurons in brain. *Nature* **408**: 860-4.

Olmo MT, Sanchez-Jimenez F, Medina MA, Hayashi H. (2002) Spectroscopic analysis of recombinant rat histidine decarboxylase. *J Biochem (Tokyo)* **132**: 433-9.

Ortiz J, Gomez J, Torrent A, Aldavert M, Blanco I. (2000) Quantitative radioisotopic determination of histidine decarboxylase using high-performance liquid chromatography. *Anal Biochem.* **280**: 111-7.

Pillot C, Heron A, Cochois V, Tardivel-Lacombe J, Ligneau X, Schwartz JC, Arrang JM (2002) A detailed mapping of the histamine H<sub>(3)</sub> receptor and its gene transcripts in rat brain. *Neuroscience* **114**: 173-93

Rittenhouse AR, Zigmond RE (1999) Role of N- and L-type calcium channels in depolarization-induced activation of tyrosine hydroxylase and release of norepinephrine by sympathetic cell bodies and nerve terminals. *J Neurobiol.* **40**: 137-48.

Rodriguez-Caso C, Rodriguez-Agudo D, Moya-Garcia AA, Fajardo I, Medina MA, Subramaniam V, Sanchez-Jimenez F. (2003) Local changes in the catalytic site of mammalian histidine decarboxylase can affect its global conformation and stability. *Eur J Biochem.* **270**: 4376-87.

Rotman EI, Murphy BJ, Catterall WA (1995) Sites of selective cAMP-dependent phosphorylation of the L-type calcium channel alpha 1 subunit from intact rabbit skeletal muscle myotubes. *J. Biol. Chem.* **270**: 16371-7.

Sunahara RK, Dessauer CW, Gilman AG. (1996) Complexity and diversity of mammalian adenylyl cyclases. *Annu Rev Pharmacol Toxicol.* **36**: 461-80.

Taguchi Y, Watanabe T, Kubota H, Hayashi H, Wada H. (1984) Purification of histidine decarboxylase from the liver of fetal rats and its immunochemical and immunohistochemical characterization. *J Biol Chem.* **259**: 5214-21.

Takemura M, Kishino J, Yamatodani A, Wada H. (1989) Inhibition of histamine release from rat hypothalamic slices by omega-conotoxin GVIA, but not by nilvadipine, a dihydropyridine derivative. *Brain Res* **496**: 351-6.

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

Tamse CT, Xu Y, Song H, Nie L, Yamoah EN (2003) Protein kinase A mediates voltage-dependent facilitation of Ca<sup>2+</sup> current in presynaptic hair cells in *Hermissenda crassicornis*. *J Neurophysiol* **89**: 1718-26.

Tanaka T, Ohmura T, Hidaka H. (1982) Hydrophobic interaction of the Ca<sup>2+</sup>-calmodulin complex with calmodulin antagonists. Naphthalenesulfonamide derivatives. *Mol Pharmacol.* **22**: 403-7.

Tang WJ, Gilman AG. (1991) Type-specific regulation of adenylyl cyclase by G protein beta gamma subunits. *Science* **254**: 1500-3.

Tokumitsu H, Chijiwa T, Hagiwara M, Mizutani A, Terasawa M, Hidaka H. (1990) KN-62, 1-[N,O-bis(5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine, a specific inhibitor of Ca<sup>2+</sup>/calmodulin-dependent protein kinase II. *J Biol Chem.* **265**: 4315-20.

Yamauchi T, Nakata H, Fujisawa H. (1981) A new activator protein that activates tryptophan 5-monooxygenase and tyrosine 3-monooxygenase in the presence of Ca<sup>2+</sup>-, calmodulin-dependent protein kinase. Purification and characterization. *J Biol Chem.* **256**: 5404-9.

## FOOTNOTES

This work was supported by spanish grants BFI 2002-01077 (MCyT), 98/119-00 (La Caixa Foundation) to I.Blanco, SAF2002/2586 (MCyT) and PAI-CVI267 (Junta de Andalucia) to F. Sánchez-Jiménez.. A. Torrent was recipient of a fellowship from the Universitat Autònoma de Barcelona.

Send reprint requests to:

Jordi Ortiz, PhD

Universitat Autònoma de Barcelona

Dept. Biochemistry & Molecular Biology

School of Medicine, Neuroscience Institute, Room M2-120

E-08193 Bellaterra (Spain)

Email: Jordi.Ortiz@uab.es

Phone +34 677 392 828 (cellular) / +34 935 811 625

Fax: +34 935 811 573

Author's address:

Universitat Autònoma de Barcelona, Neuroscience Institute, Dept. Biochemistry & Molecular Biology, Bellaterra, Spain (A.T., D.M., J.G., I.B., and J.O.) and Universidad de Málaga, Dept. Molecular Biology & Biochemistry, Málaga, Spain (D.R., C.R., and F.S.)



## FIGURE LEGENDS

Figure 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 [<sup>3</sup>H]-histidine and depolarization with 30 mM KCl. 3 μM omega-conotoxin MVIIC inhibits N-, Q-, and P-type calcium channels while at 0.1 μM it only inhibits Q-type channels. Omega-conotoxin GVIA blocks N-type calcium channels. Results represent means ± SEM. Number of slice incubations are indicated between brackets above the columns. Mean 100 % of histamine synthesis in 2mM K<sup>+</sup> controls was 18 fmol mg protein<sup>-1</sup> h<sup>-1</sup>. \*, p<0.01 compared with controls (2mM K<sup>+</sup>). #, p<0.01 compared with 30 mM K<sup>+</sup>, one-way ANOVA plus Bonferroni post-hoc tests.

Figure 2. Inhibition of calcium/calmodulin-dependent protein kinase II (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 [<sup>3</sup>H]-histidine was added to the incubation buffer and the slices were depolarized with 30 mM KCl. Results are means ± SEM. Number of slice incubations are indicated in brackets above the columns. Mean 100 % of histamine synthesis in 2mM K<sup>+</sup> controls was 17 fmol mg protein<sup>-1</sup> h<sup>-1</sup>. \*, p<0.001 compared with controls (2mM K<sup>+</sup>); #, p<0.001 compared with 30 mM K<sup>+</sup>, one or two way ANOVA plus Bonferroni post-hoc test.

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

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

Figure 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<sub>14-22</sub> (10  $\mu$ M) suppressed IBMX effects. All inhibitors except IBMX were added to the incubation 20 minutes before [<sup>3</sup>H]-histidine. IBMX was added 15 min before [<sup>3</sup>H]-histidine. Results represent means  $\pm$  SEM. Number of slice incubations are indicated in brackets above the columns. Mean 100% of histamine synthesis in 2mM K<sup>+</sup> controls was 15 fmol mg protein<sup>-1</sup> h<sup>-1</sup>. \*, P<0.01 compared with controls ( 2mM K<sup>+</sup> ); #, p<0.01 compared to IBMX. B, IBMX effects are additive to those of depolarization. IBMX was added 15 min before [<sup>3</sup>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<sup>-1</sup> h<sup>-1</sup>. \*, p<0.01 compared with basal values; #, P<0.01 compared with 30 mM K<sup>+</sup> or IBMX stimulation. C, IBMX effects are prevented by incubation with the H<sub>3</sub> agonist imetit, even in the presence of omega-conotoxin GVIA. Imetit and GVIA were added 20 min before [<sup>3</sup>H]-histidine. IBMX was added 15 min before [<sup>3</sup>H]-histidine. Mean 100% 2 mM K<sup>+</sup> controls were 14 fmol mg protein<sup>-1</sup> h<sup>-1</sup>. \*, p<0.01 vs. basal values; #, p<0.01 vs. IBMX.

Figure 6. Protein kinase A phosphorylates and activates recombinant 1/512 histidine decarboxylase (HDC) in vitro. A, autoradiogram showing the incorporation of <sup>32</sup>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 His and 0.1  $\mu$ M [<sup>3</sup>H]-His after PKA phosphorylation *in vitro*. Results are means  $\pm$  SEM. Number of determinations is indicated in brackets above the columns. Mean 100% of HDC activity was 1482 nmol mg protein<sup>-1</sup> h<sup>-1</sup>. \*, p<0.001 two-tailed Student t-test.

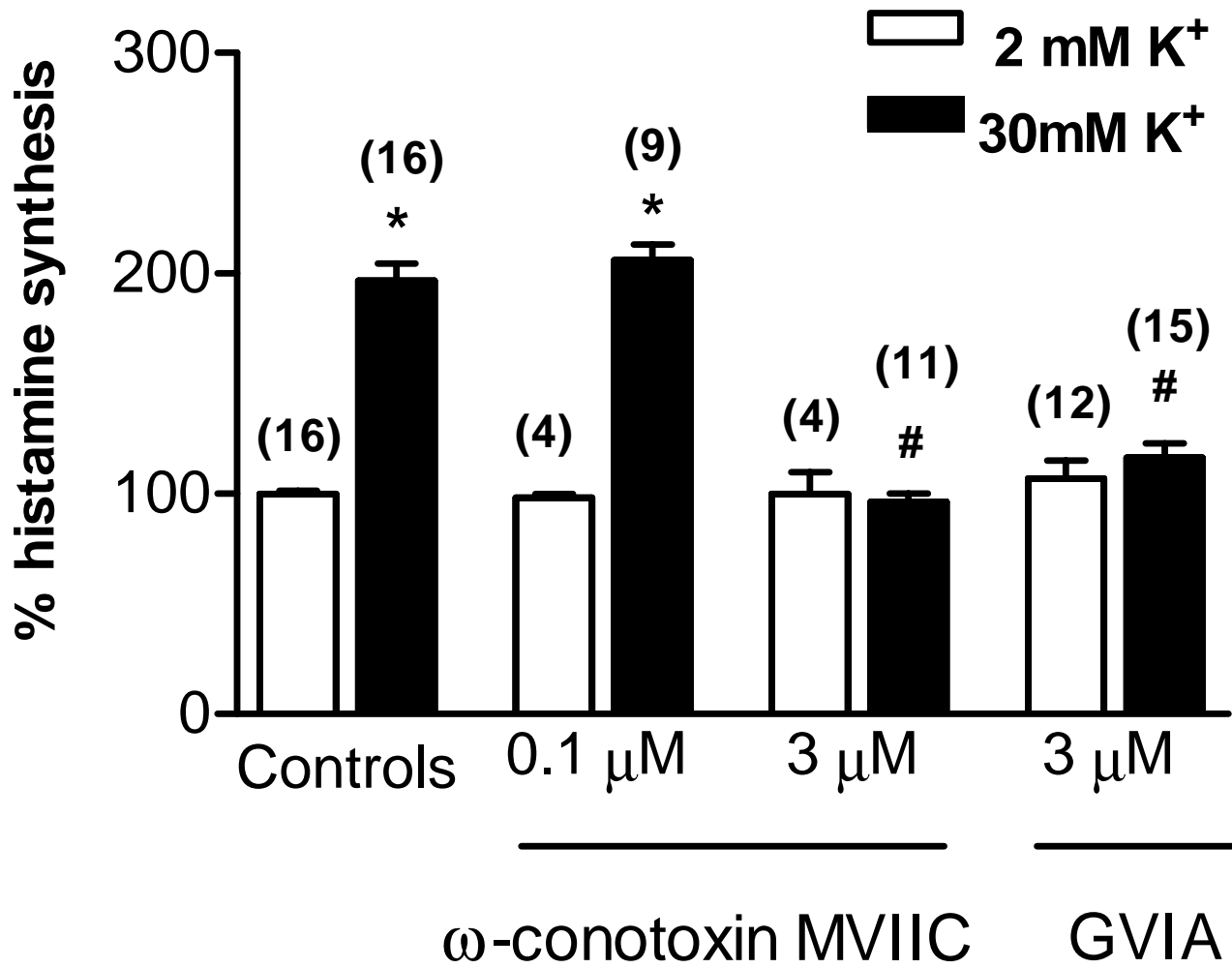


Figure 1

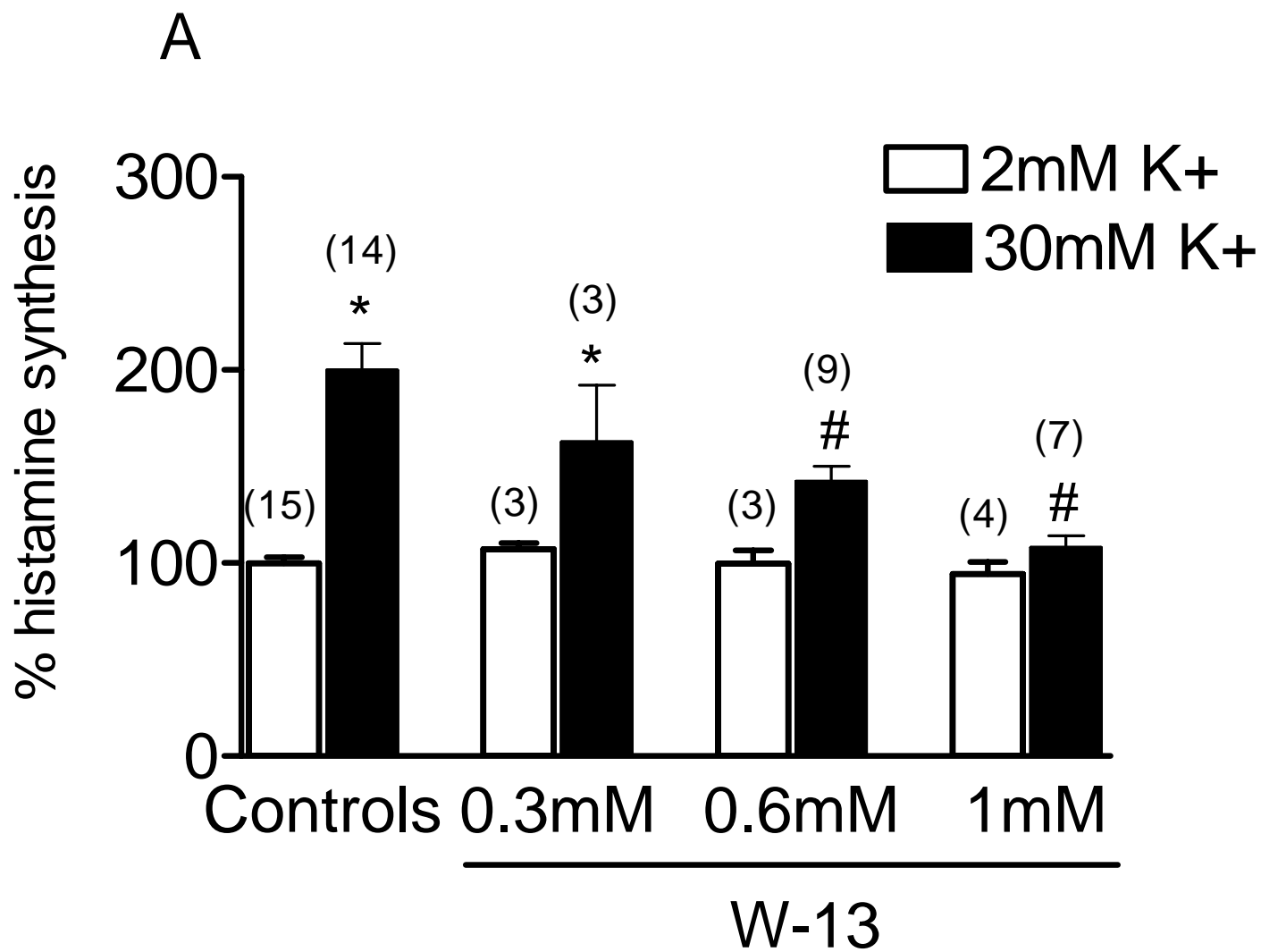


Figure 2A

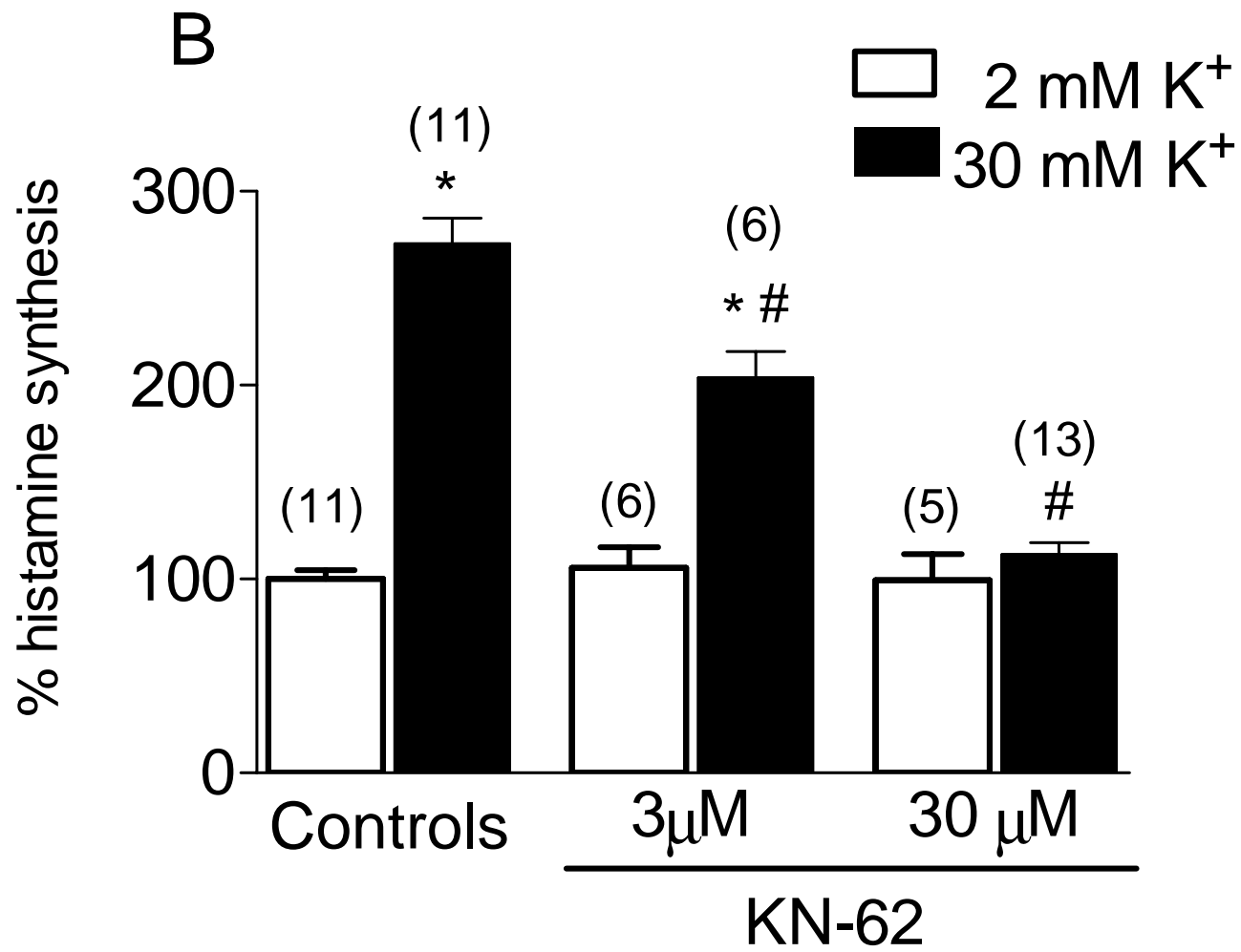


Figure 2B

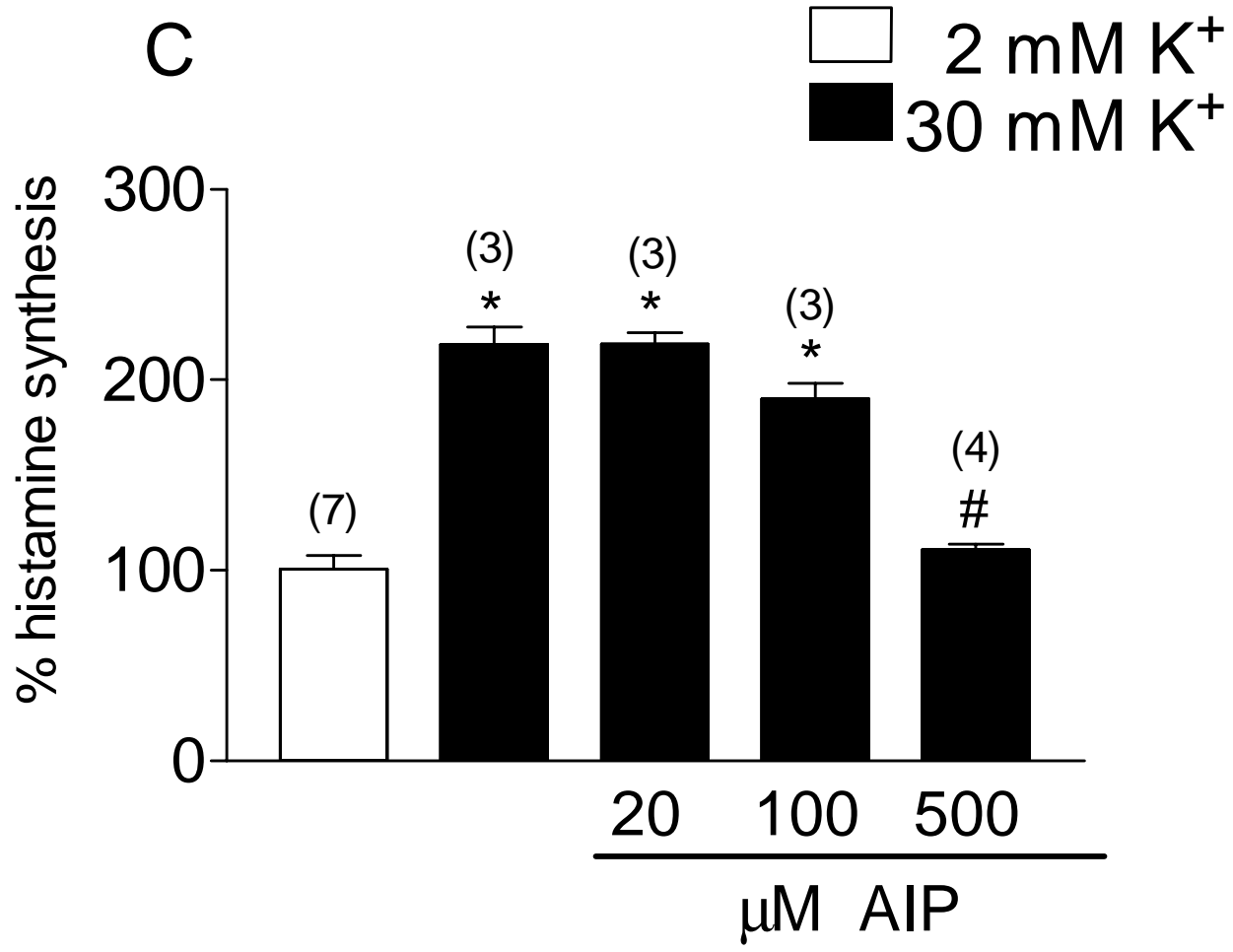
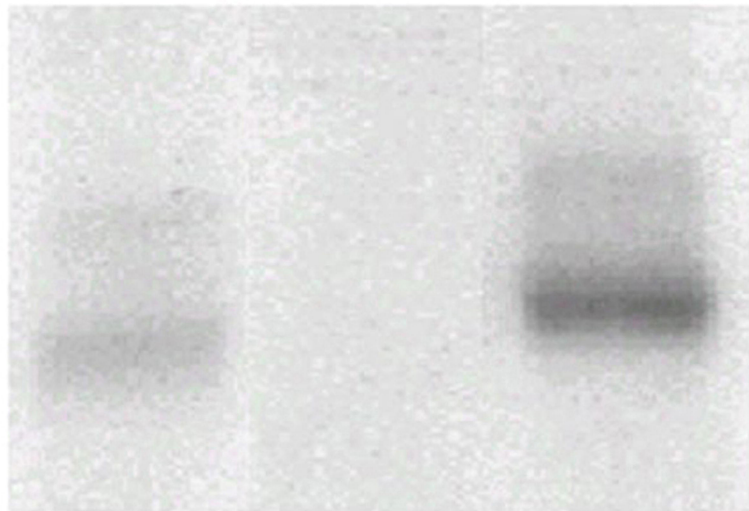


Figure 2C

# A

55 kDa -



HDC

-

+

+

CaMKII

+

-

+

Figure 3A



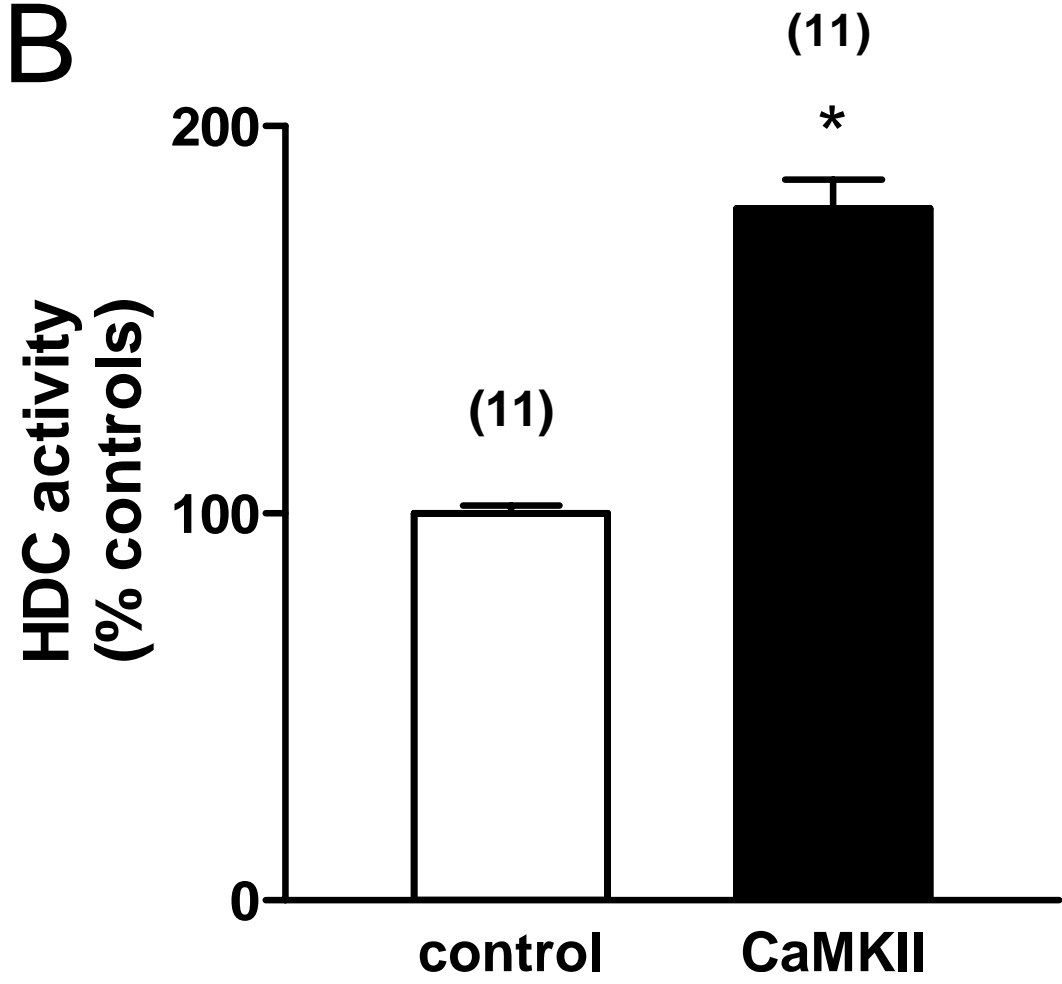


Figure 3B

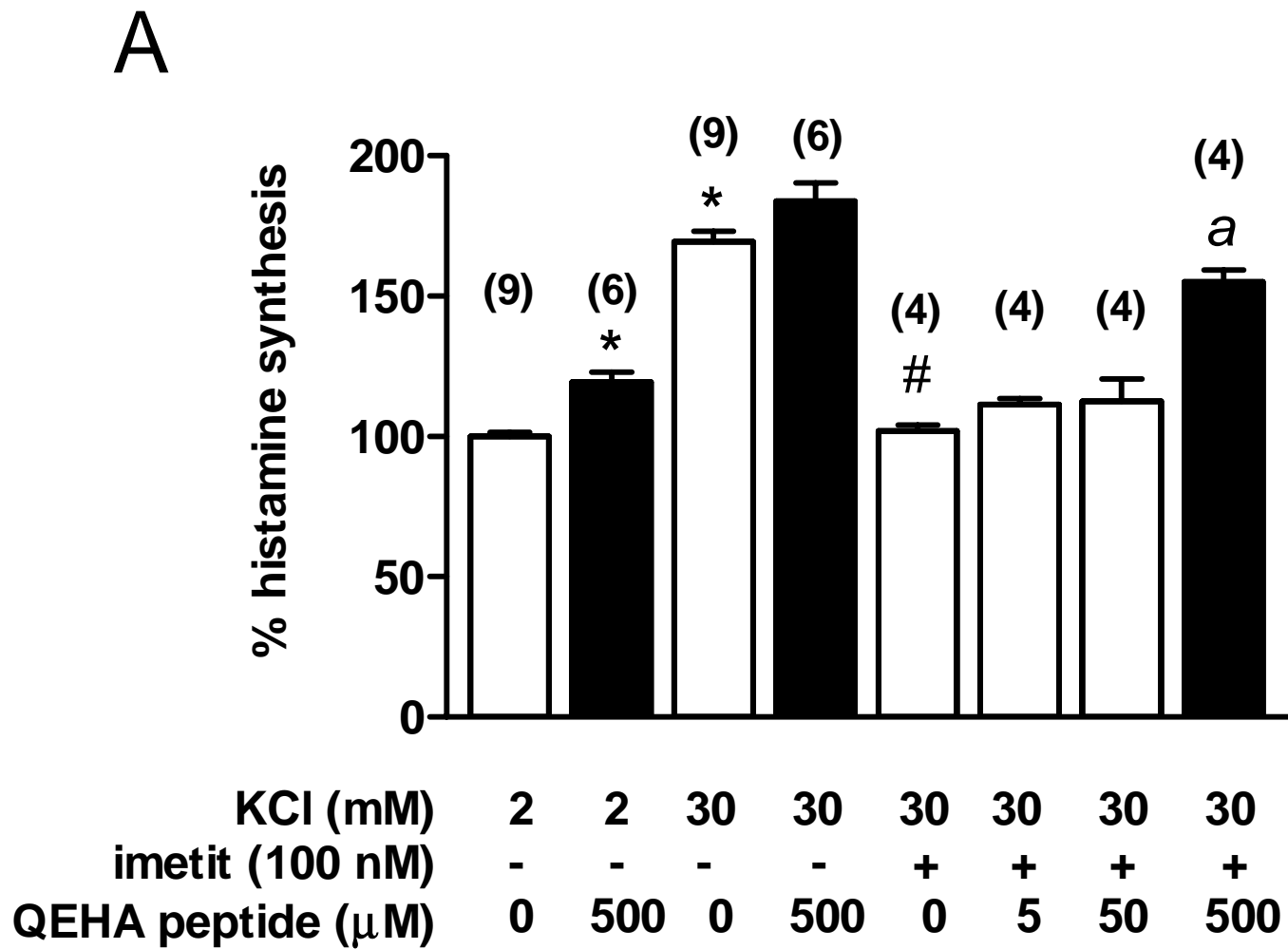


Figure 4A

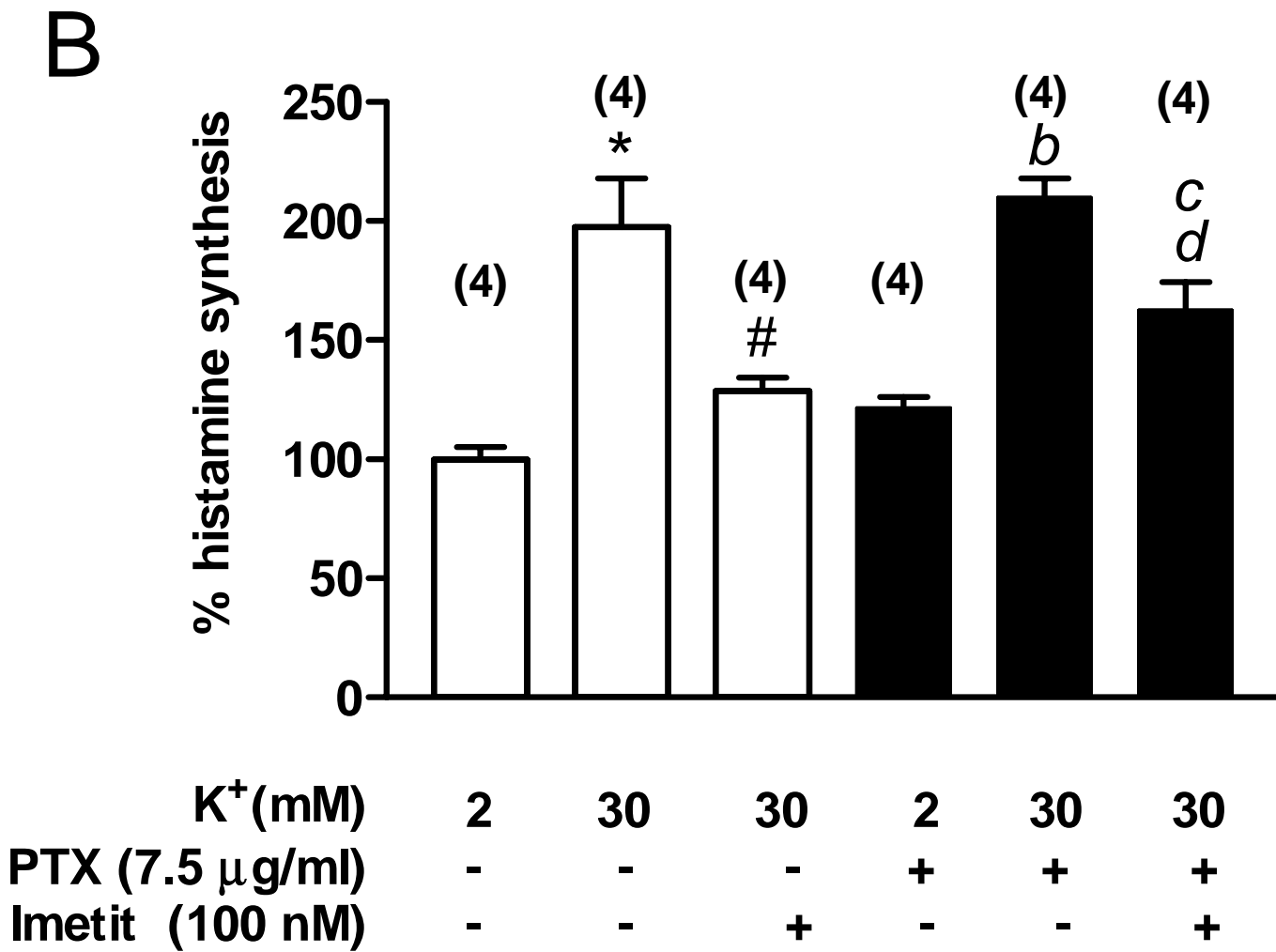


Figure 4B

C

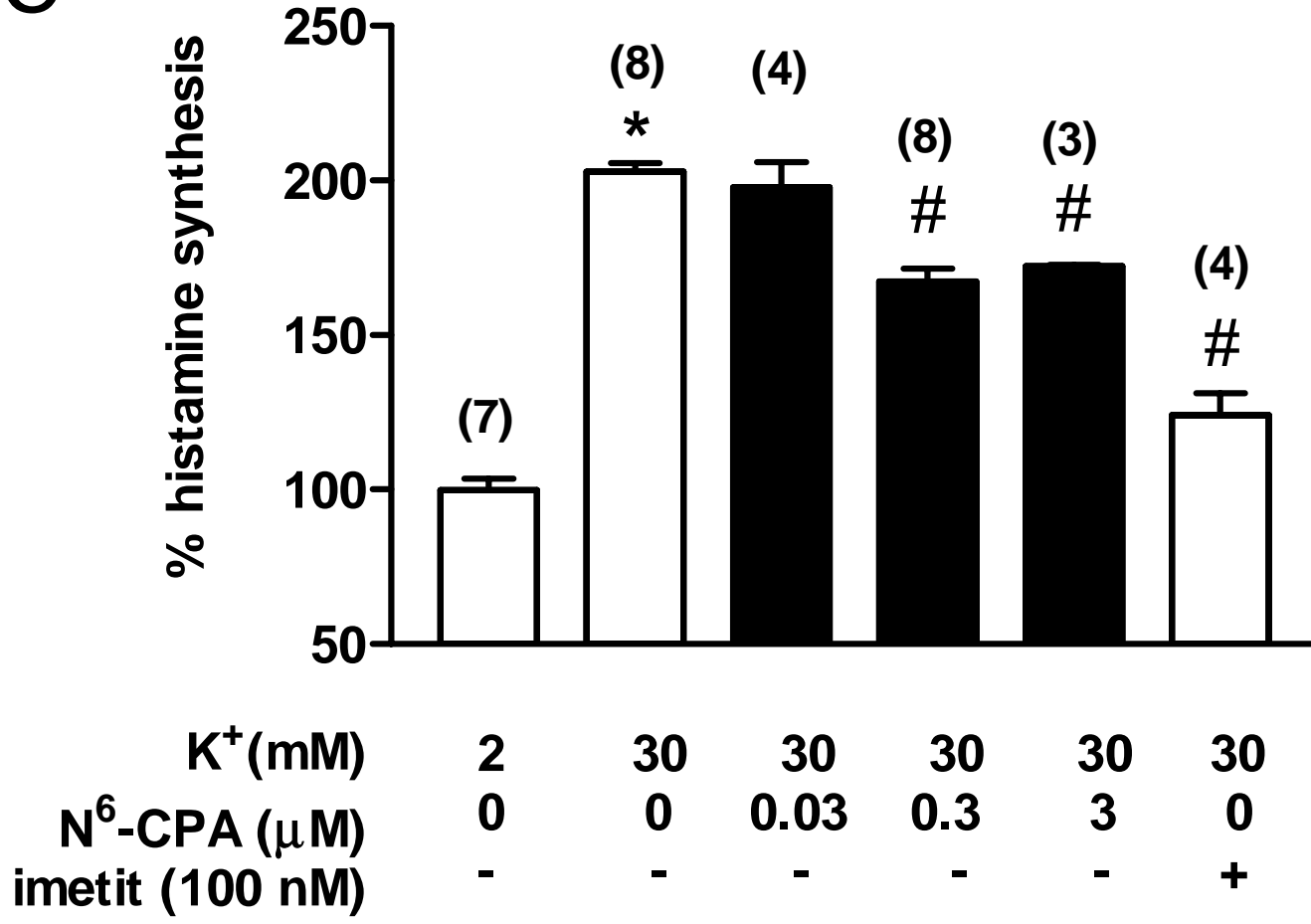


Figure 4C

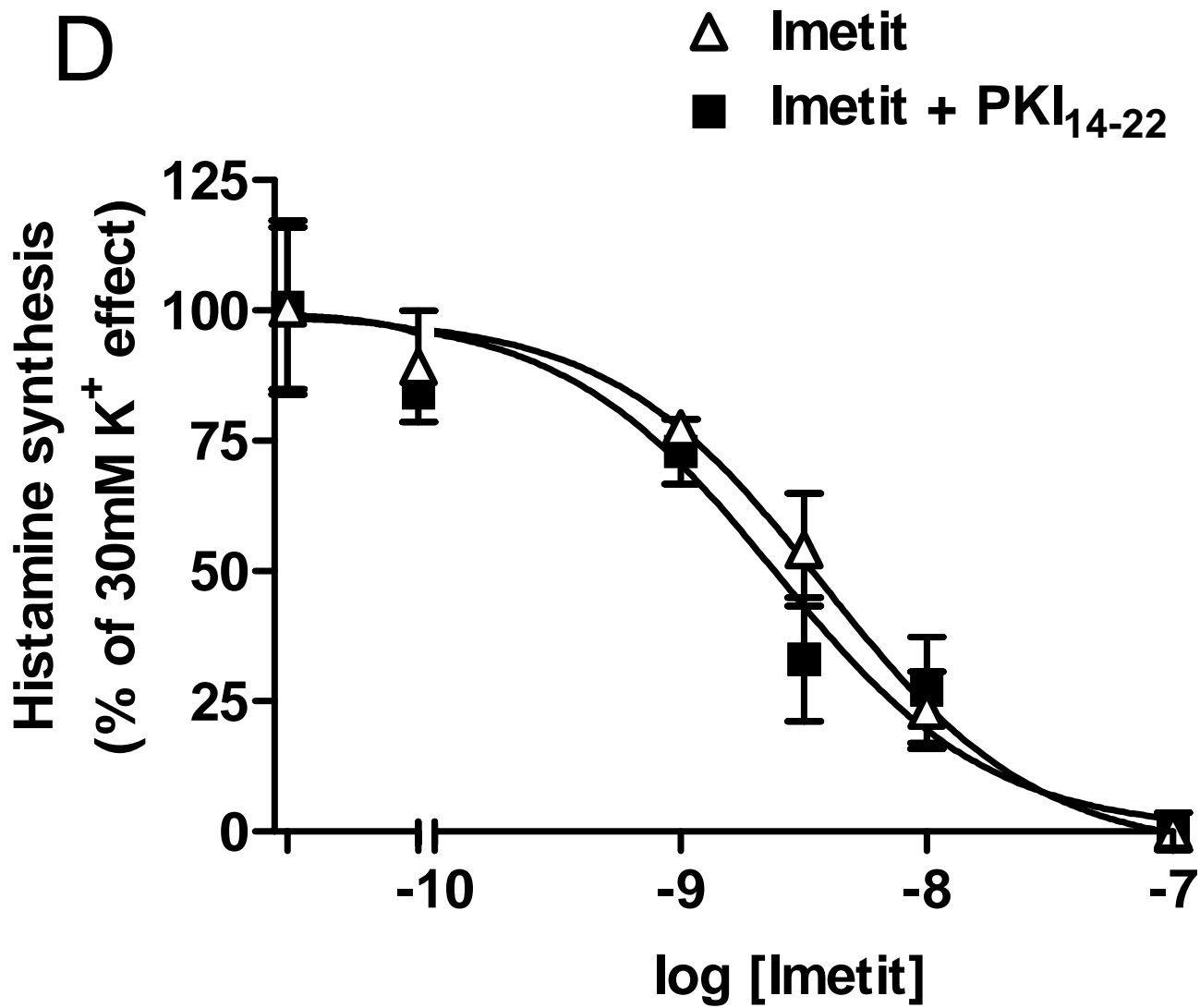


Figure 4D

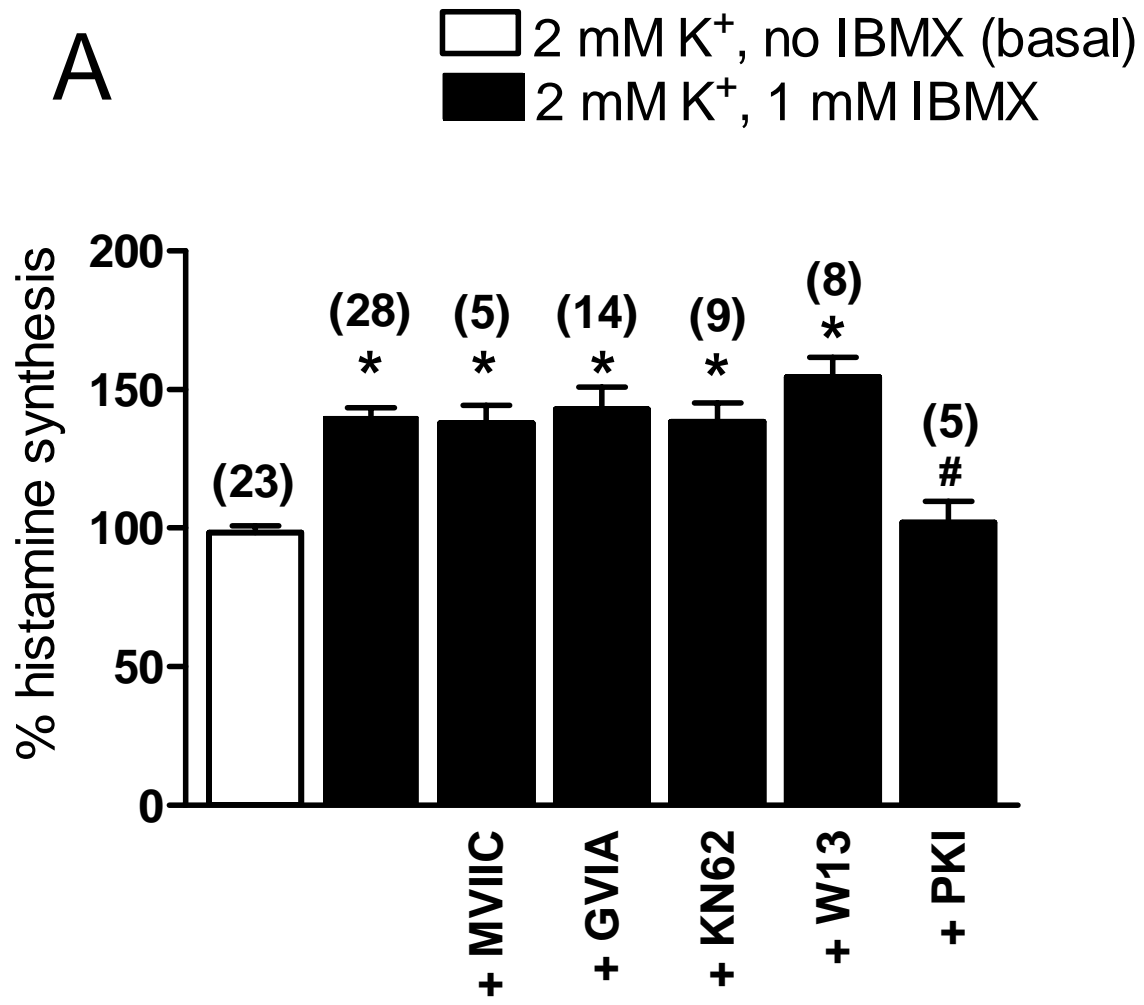


Figure 5A

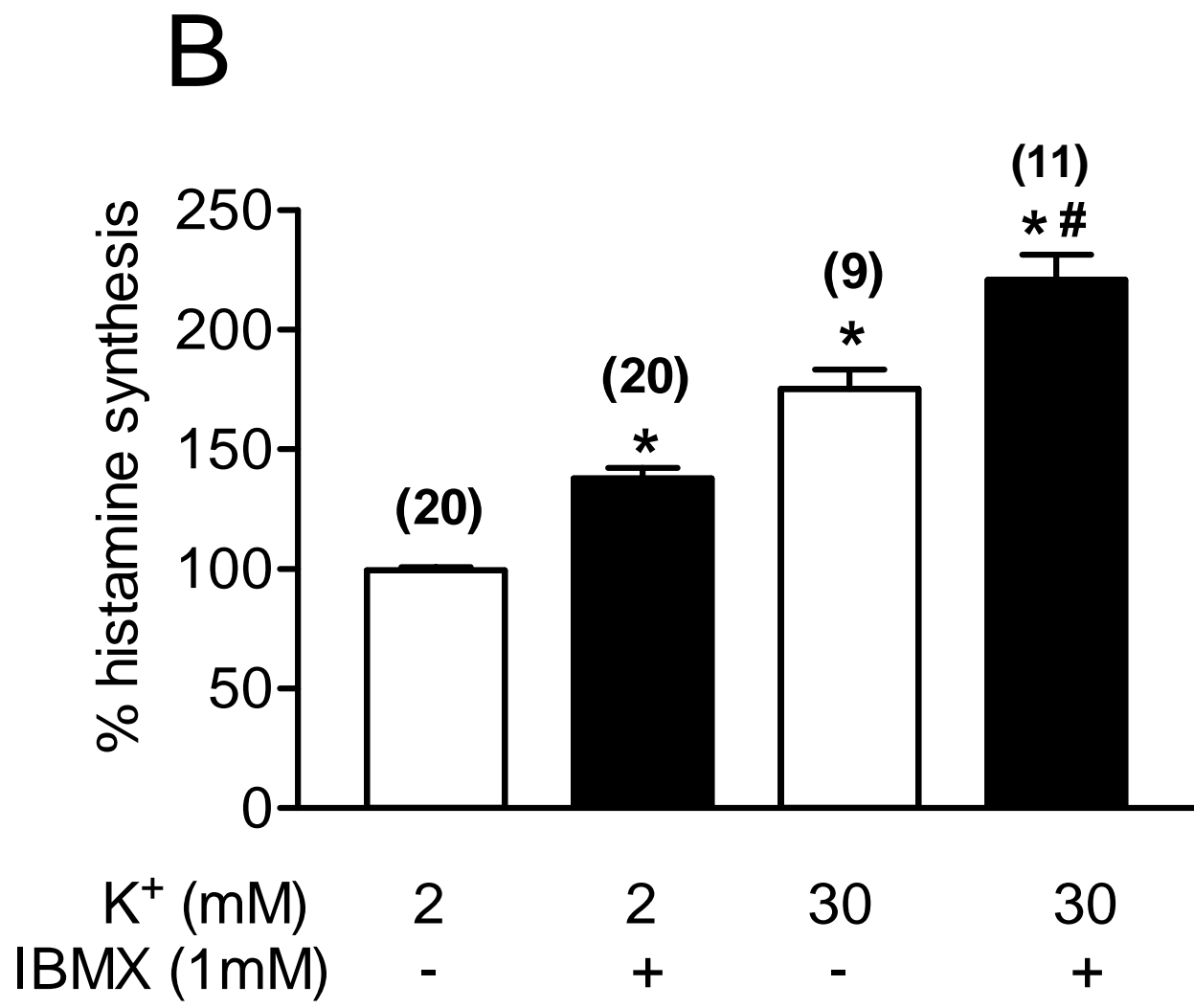


Figure 5B

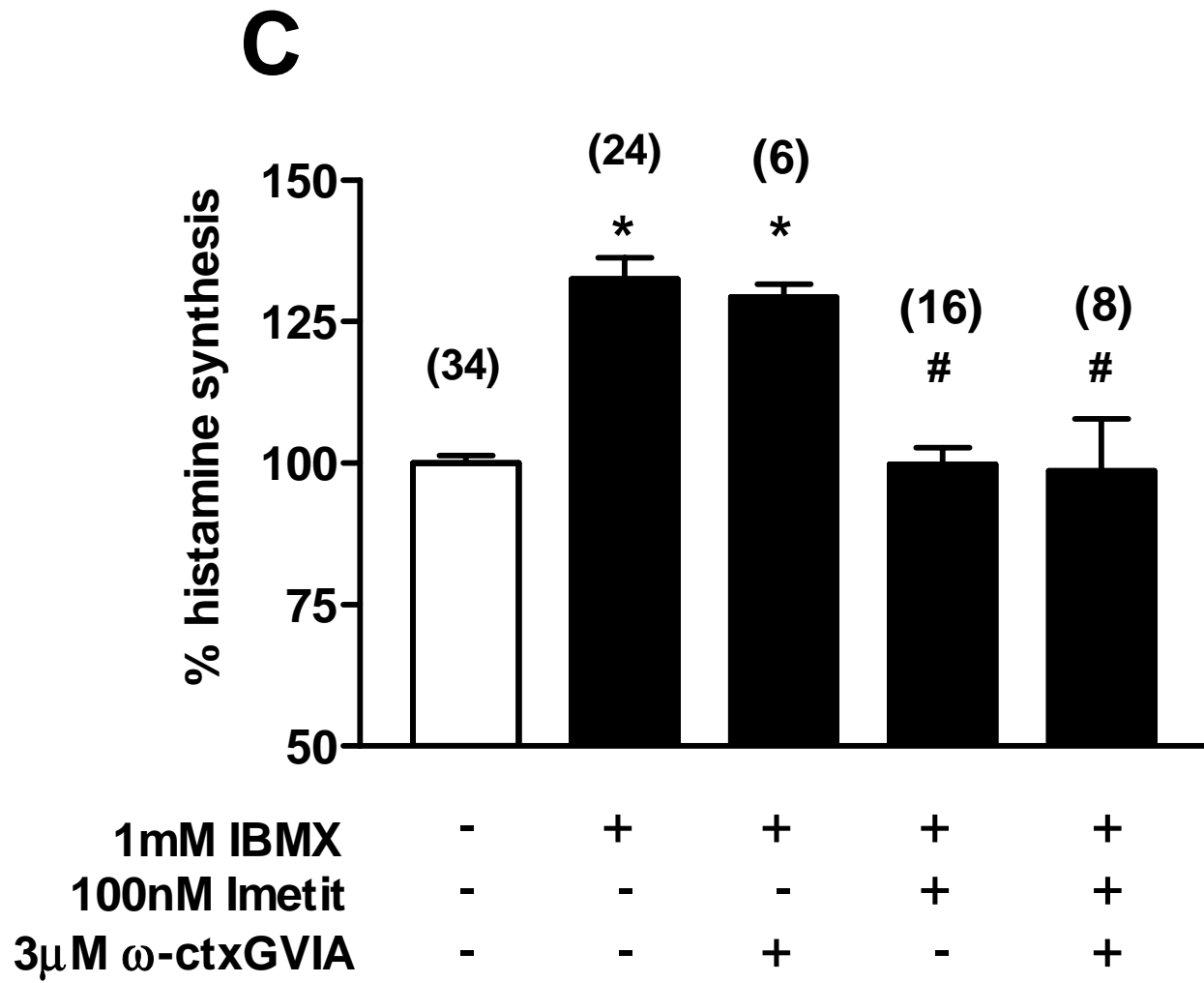


Figure 5C



# A

55 kDa -



HDC

+

-

+

PKA

+

+

-

Figure 6A

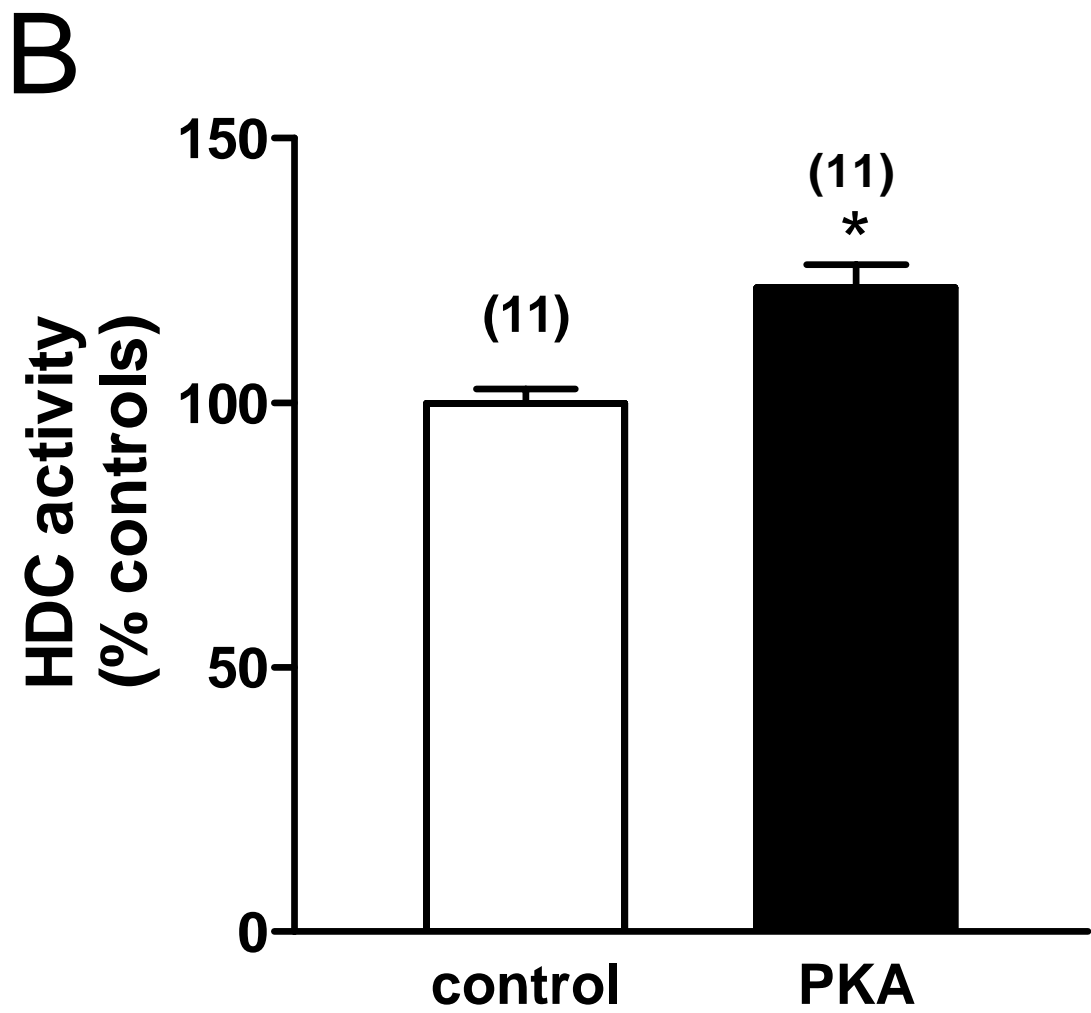


Figure 6B