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H_3 AUTORECEPTORS MODULATE HISTAMINE SYNTHESIS THROUGH CALCIUM/CALMODULIN- AND cAMP-DEPENDENT PROTEIN KINASE PATHWAYS

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Running title: Modulation of histamine synthesis by H_3 receptors

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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_{14-22} = 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.

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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 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 $G_{i/o}$-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.

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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_1 to H_4) have been identified and cloned. The H_3 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_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 MAP kinase phosphorylation in receptor-transfected cells (Drutel et al., 2001). It has been shown that H_3 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_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 enzyme synthesizing histamine, histidine decarboxylase (HDC). HDC is a pyridoxal-5'-phospate 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_3 receptors (Gomez-Ramirez et al., 2002). As protein kinase A (PKA) activation was required also for the effects of H_3 receptor inverse agonists, we concluded that this pathway mediated H_3 autoreceptor 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 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_3 receptors represses the effects elicited through either of these kinases, which apparently are mutually independent.

MATERIALS AND METHODS

Chemicals: Ring-labelled [2,5-^{3}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_{14-22}, 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, Findland).

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 $CaCl_2$, 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 (4ºC), 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 pippetted into 2 ml polypropylene tubes.

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Up to 24 tubes containing slices were obtained from a single rat brain cortex. The tubes were preincubated for 25 min at 37ºC in a Eppendorf Thermomixer under O_2/CO_2 (95:5) atmosphere. Previously purified [^{3}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 [^{3}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 [^{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, 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 [^{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 4ºC. 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 ºC) and supernatants recovered and processed as follows in [^{3}H]-Histamine Purification by HPLC.

[^{3}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 % [^{3}H]-histidine, increasing the sensitivity of subsequent [^{3}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

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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 [^{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 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). [^{3}H]-histamine synthesis in each sample was estimated from dpm obtained corrected by recovery, blank dpm and specific activity of [^{3}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 ºC 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

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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 $CaCl_2$, 5 mM $MgCl_2$ and 50 µM ATP (containing 10 µCi gamma-^{32}P-ATP; Amersham) in a total volume of 100 µl. Incubations were carried out for 40 min at 30 ºC 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 $CaCl_2$ 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 Precission Plus^{TM}. After Coomassie blue stain the gels were dried over vacuum for 30 min at 70 ºC 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-^{32}P-ATP was omitted. Thereafter, phosphorylated HDC and control samples were incubated in 50 mM HEPES buffer pH 7.4, 5 mM MgCl2, 10 µM pyridoxal-5'-phosphate and 60 µM histidine (0.1 µM [^{3}H]-His of 43 Ci/mmol) in a final volume of 100 µl. Samples were incubated for 30 min at 37 ºC and [^{3}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 appropiate controls. Statistical significance of differences between values was evaluated by analysis of variance (ANOVA) followed by Bonferroni post-hoc tests for multiple group comparisons.

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RESULTS

In preliminary experiments we observed that histamine synthesis in rat brain cortical slices could be stimulated by depolarization with either K^+ or 4-aminopirydine 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 μM 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 μM) 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-

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13 may appear relatively high as compared to the IC50 of calmodulin inhibition in homogenates (about 60 μM, 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 autocamtide-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_{50} 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^{2+}, calmodulin and gamma-^{32}P-ATP resulted in strong ^{32}P incorporation, as determined by autoradiograhy of vacuum-dried SDS-PAGE gels. ^{32}P labelled a protein of Mr 55,000, the expected size of 1/512 HDC (Figure 3A, right lane). Incubation controls containing CaMKII, Ca^{2+}, calmodulin and gamma-^{32}P-ATP but lacking purified HDC showed a minor incorporation of ^{32}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

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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 μM His (0.1 μM [^{3}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 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 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 betagama subunits on several effectors such as adenylate cyclases, G protein-regulated kinase and a 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 $G_{i/o}$ protein subunits were involved in the effects of imetit, we incubated slices in the presence of 7.5 μg/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 %

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(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, α_2-adrenergic, $GABA_B$ or $5\text{-}HT_{1B}$ 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 maximun 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 $PKI_{14\text{-}22}$ at 10 μM (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 $PKI_{14\text{-}22}$ (10 μM; Figure 5A) and Rp-adenosine-3'5'-cyclic monophosphate (500 μM; 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

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inhibitor of calcium entry or CaMKII tested altered significantly IBMX effects (3 μM omega-conotoxin MVIIC, 3 μM omega-conotoxin GVIA, 1 mM W-13 and 30 μM 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_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 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-^{32}P-ATP. Autoradiograhy of vacuum-dried SDS-PAGE gels resulted in a clear ^{32}P incorporation into the protein (Figure 6A). Incubation controls were not different from background. Furthermore, activity of phosphorylated HDC in the presence of 60 μM His (0.1 μM [^{3}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.

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DISCUSSION

This paper 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 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_3 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 tuberommamillary 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 hydrofobic 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 91-93 %

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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 impairement of [^{45}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_{50} of CaMKII inhibition for the three compounds are much lower than the concentrations needed in slices, we must note that diffusion accross membranes is a principal factor determining 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 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^{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 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.,

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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_3 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_3 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_3 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 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 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_{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), while H_3 receptors elicit a near-complete inhibition of stimulated histamine synthesis in our preparations. As H_3 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 $G_{i/o}$ 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 $G_{i/o}$-coupled receptors that would reduce depolarization effects on histamine

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synthesis. It was not surprising that adenosine A_1 receptors did, as 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 ~~is~~ inhibited by histamine H_3 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_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; Figure 4D). We show that PKA blockade did not significantly modify the inhibition curve of the H_3 agonist at different concentrations (Figure 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 betagamma 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 -hypothetically- 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 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 decissions 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_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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FOOTNOTES

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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 [^{3}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^+ ontrols was 18 fmol mg protein^{-1} h^{-1}. *, $p<0.01$ compared with controls (2mM K^+). #, $p<0.01$ compared with 30 mM K^+, 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 [^{3}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^+ controls was 17 fmol mg protein^{-1} h^{-1}. *, $p<0.001$ compared with controls (2mM K^+); #, $p<0.001$ compared with 30 mM K^+, one or two way ANOVA plus Bonferroni post-hoc test.

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Figure 3. Calcium/calmodulin-dependent protein kinase II phosphorylates and activates recombinant 1/512 histidine decarboxylkase (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 50-60 kDa undergo autophosphorylation (left lane). B, CaMKII-phosphorylated samples have higher histidine decarboxylase activity. Activity was determined in the presence of 60 μM His and 0.1 μM [^{3}H]-His after CaMKII phosphorylation in vitro. Results are means ± 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. H_3 receptor inhibits depolarization-stimulated histamine synthesis through actions of $G_{i/o}$-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 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 $G_{i/o}$-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 μM 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 ± 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 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.

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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 μM omega-conotoxin MVIIC), of N-calcium channels (3 μM omega-conotoxin GVIA), by the calmodulin inhibitor W-13 (1 mM) or by the CaMKII inhibitor KN-62 (30 μM). In contrast, the PKA inhibitor PKI_{14-22} (10 μM) suppressed IBMX effects. All inhibitors except IBMX were added to the incubation 20 minutes before [^{3}H]-histidine. IBMX was added 15 min before [^{3}H]-histidine. Results represent means ± SEM. Number of slice incubations are indicated in brackets above the columns. Mean 100% of histamine synthesis in 2mM K^+ controls was 15 fmol mg protein^{-1} h^{-1}. *, P<0.01 compared with controls (2mM K^+); #, p<0.01 compared to 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}. *, 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}. *, p<0.01 vs. basal values; #, p<0.01 vs. IBMX.

Figure 6. Protein kinase A phosphorylates and activates recombinant 1/512 histidine decarboxylkase (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 μM His and 0.1 μM [^{3}H]-His after PKA phosphorylation *in vitro*. Results are means ± SEM. Number of determinations is indicated in brackets above the columns. Mean 100% of HDC activity was 1482 nmol mg protein^{-1} h^{-1}. *, p<0.001 two-tailed Student t-test.

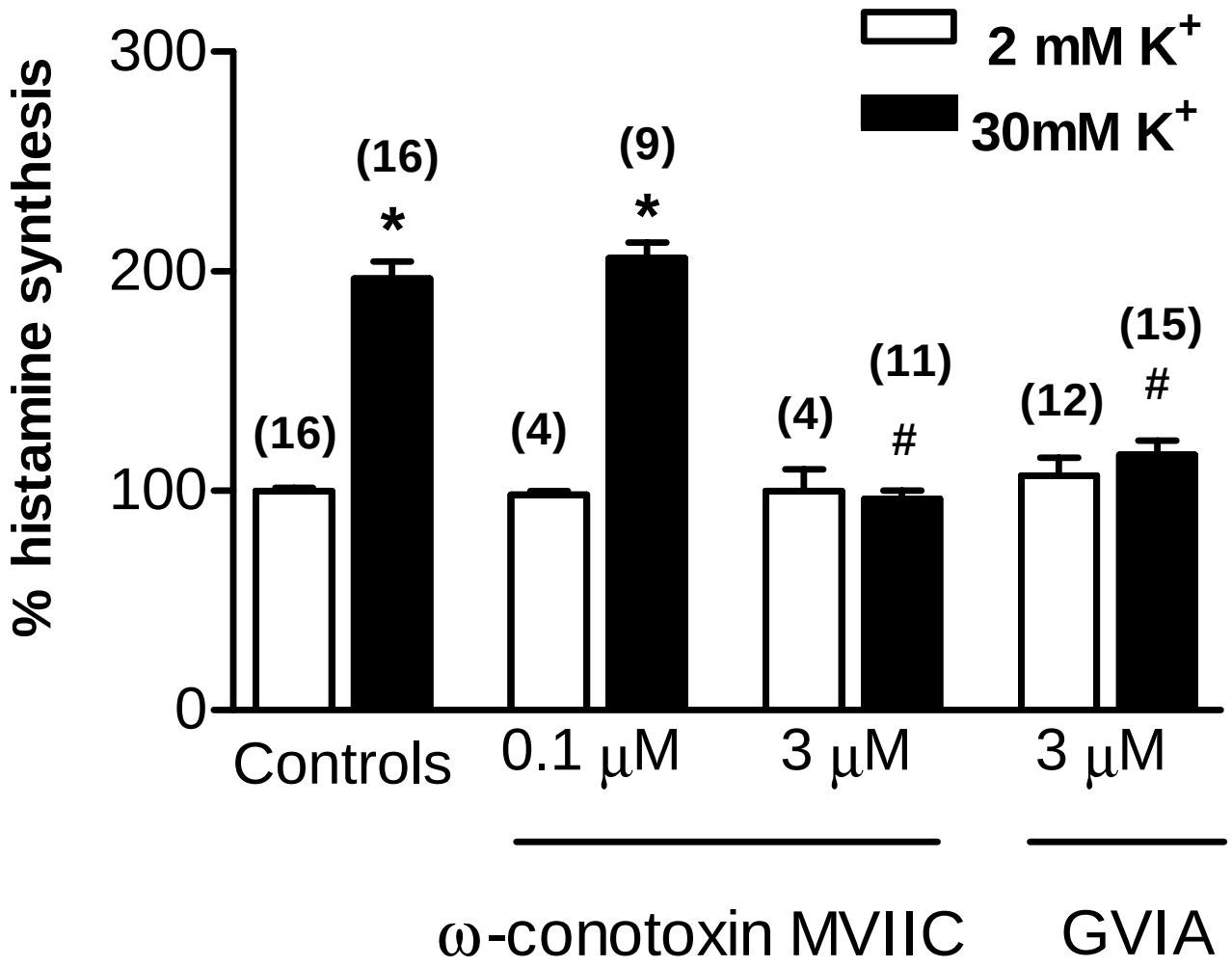


Figure 1

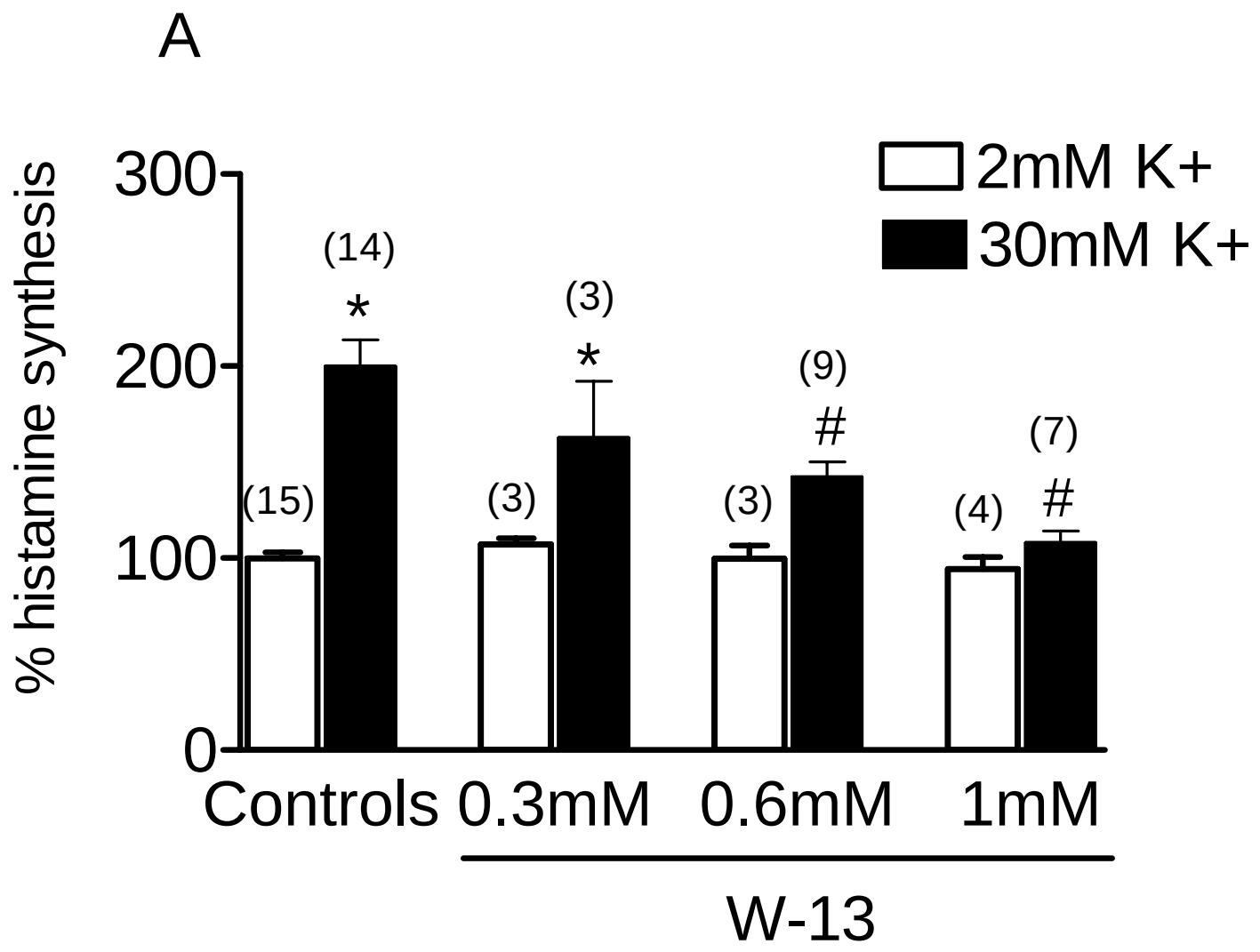


Figure 2A

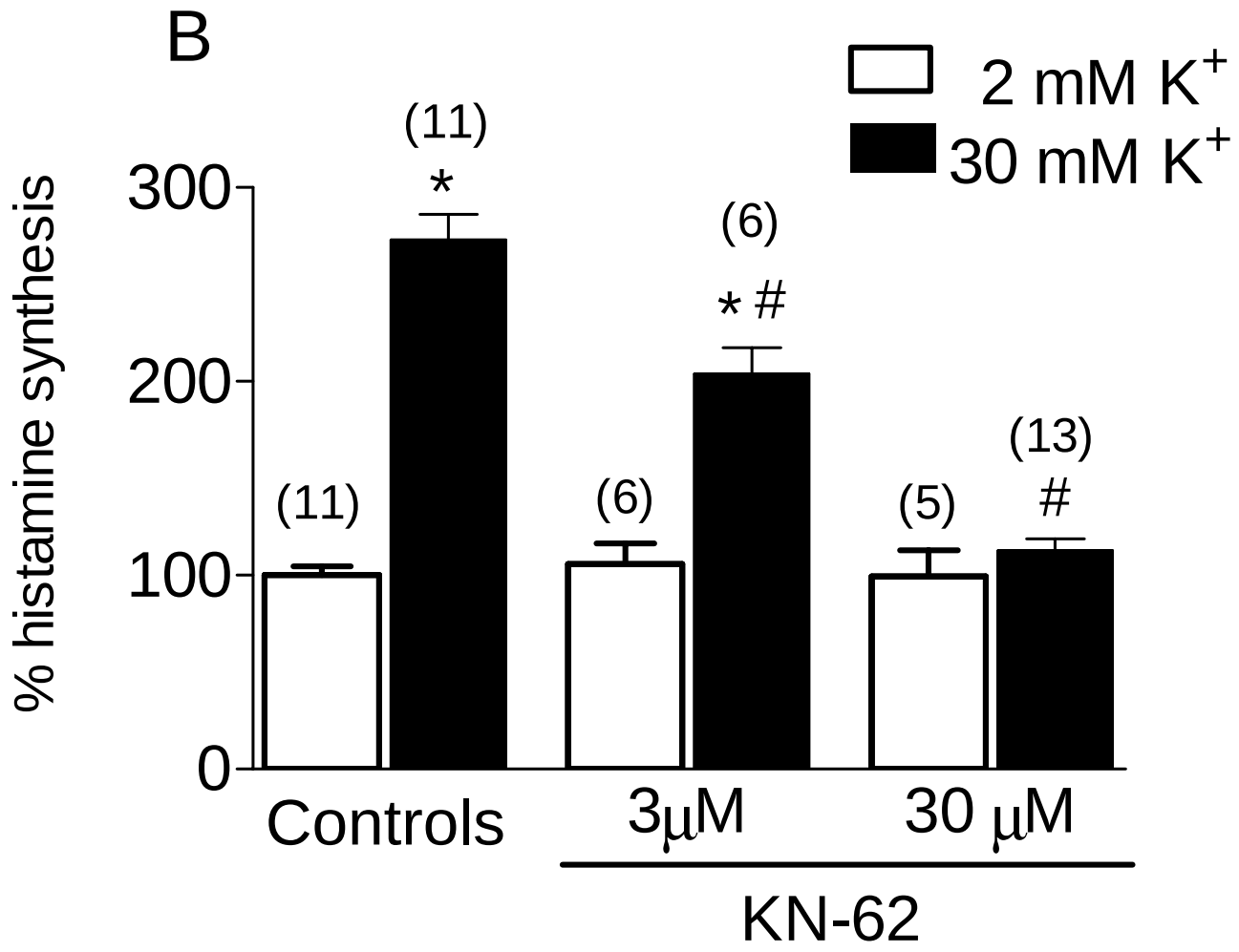


Figure 2B

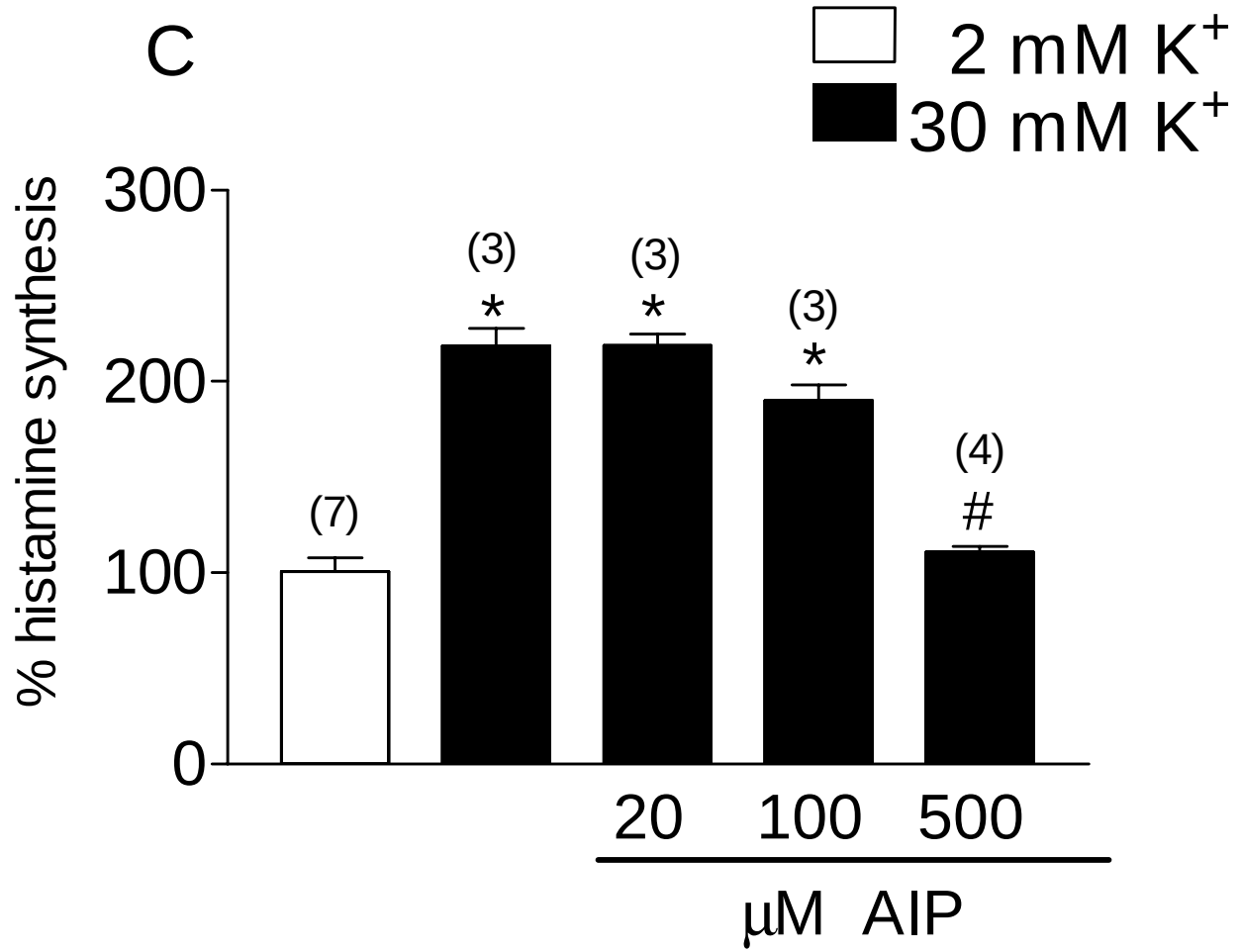


Figure 2C

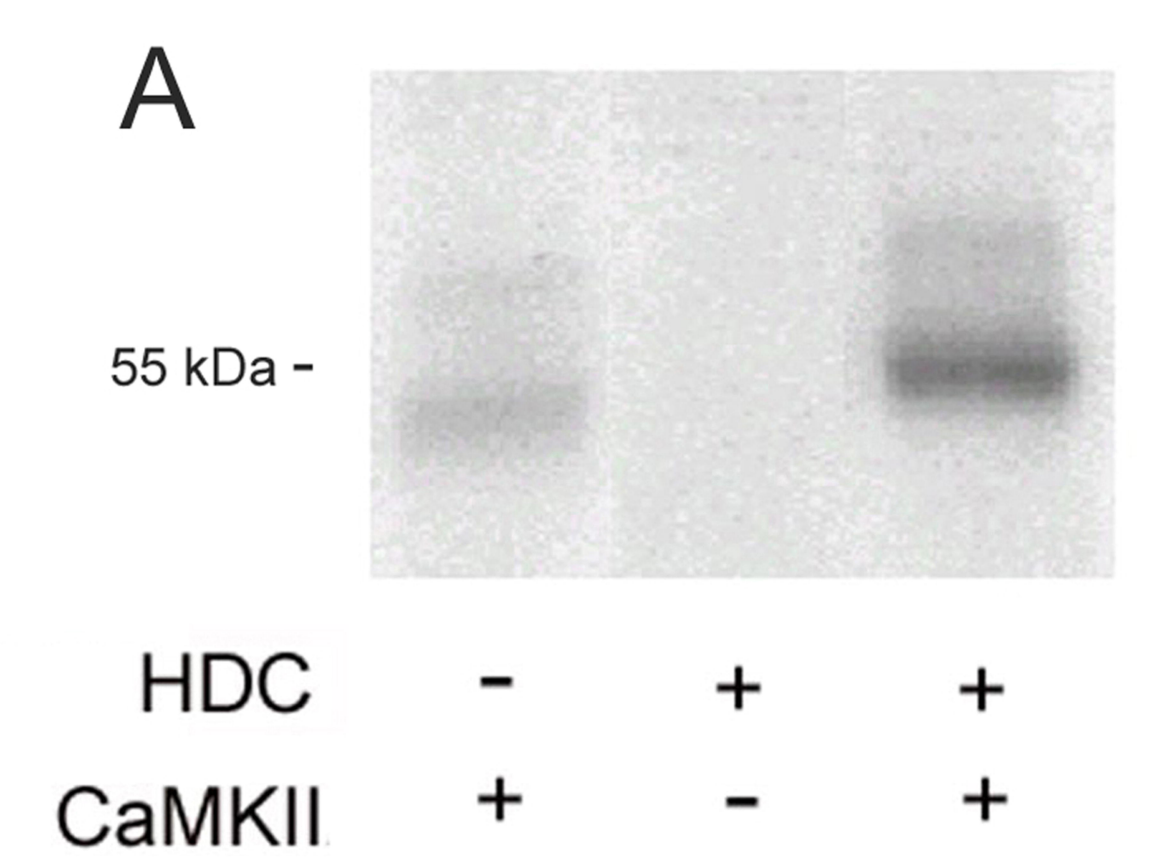


Figure 3A

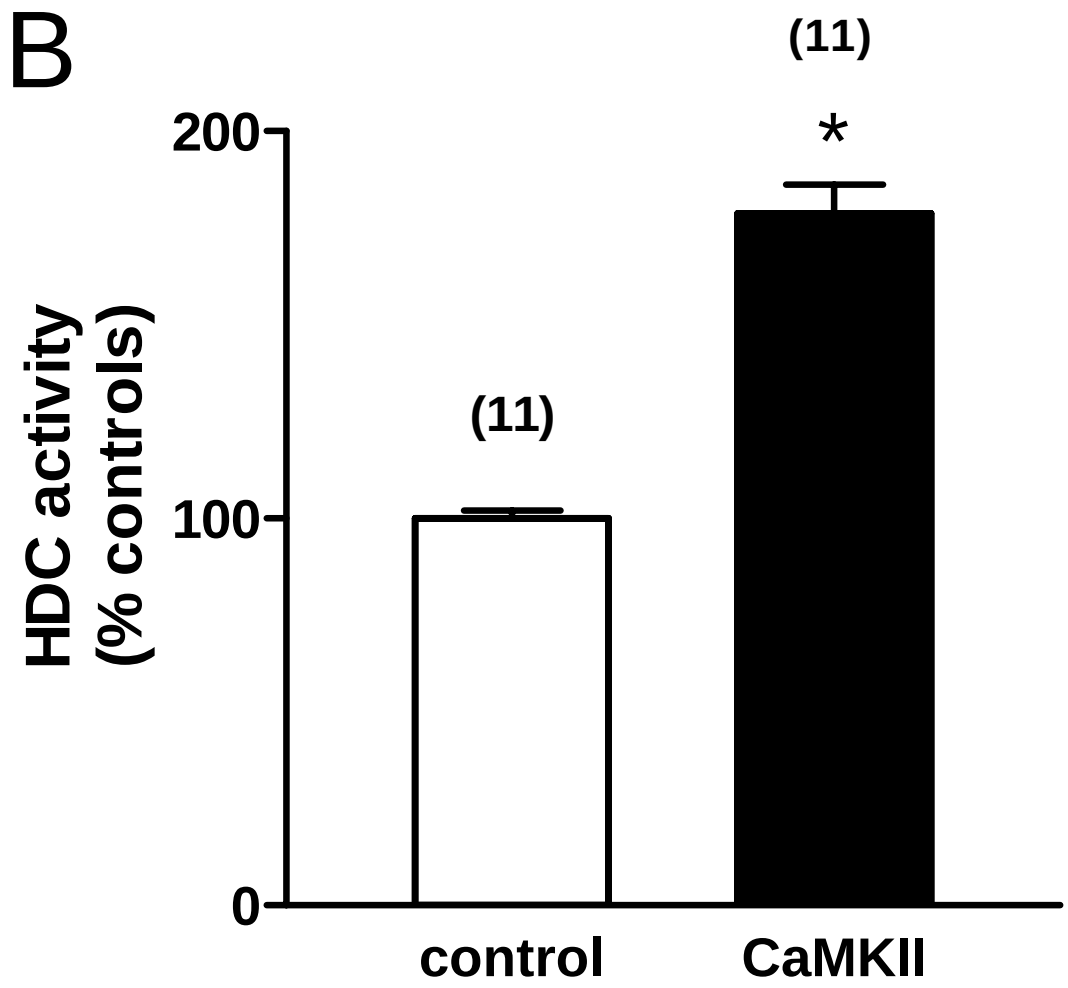


Figure 3B

A

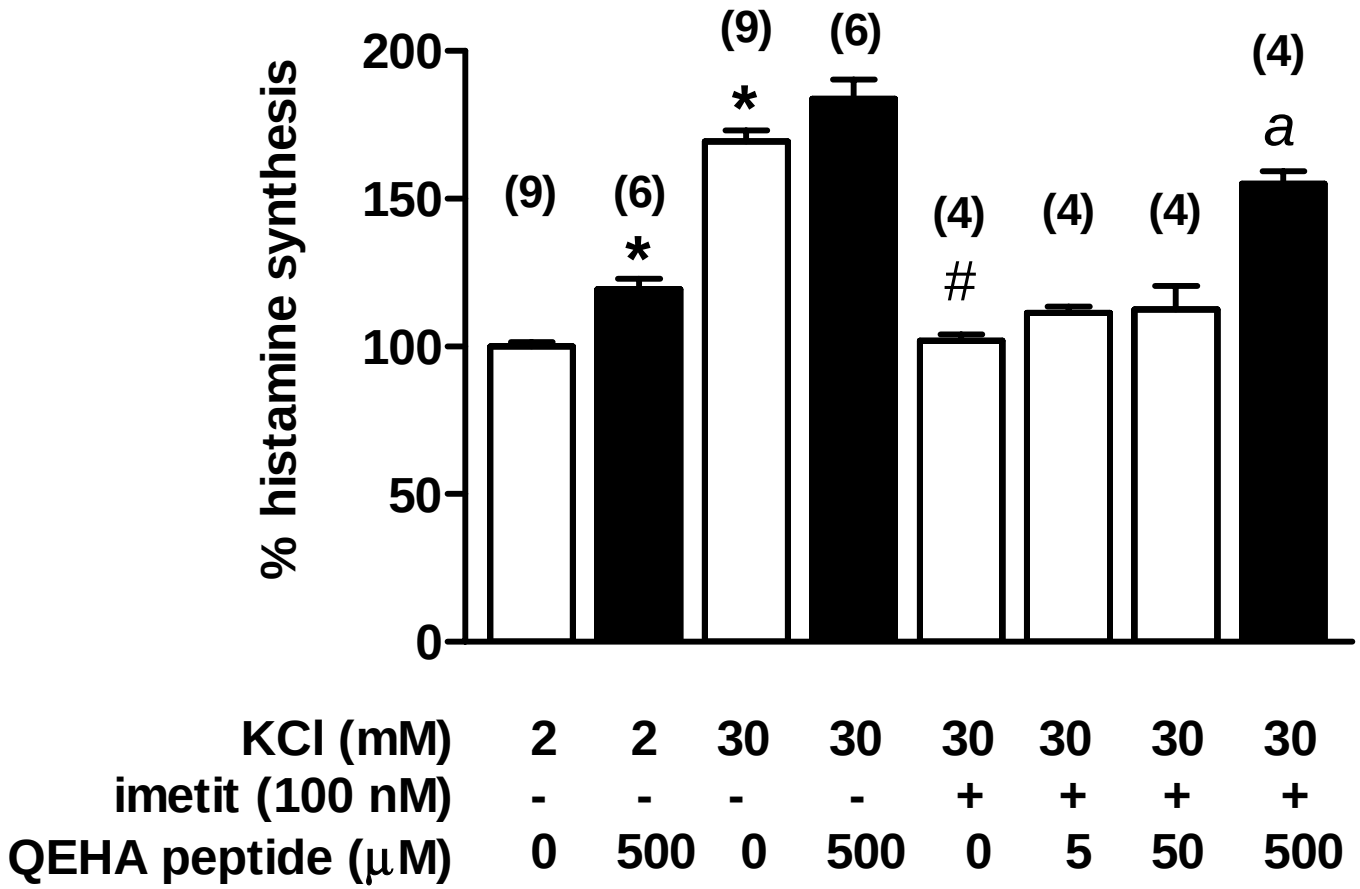


Figure 4A

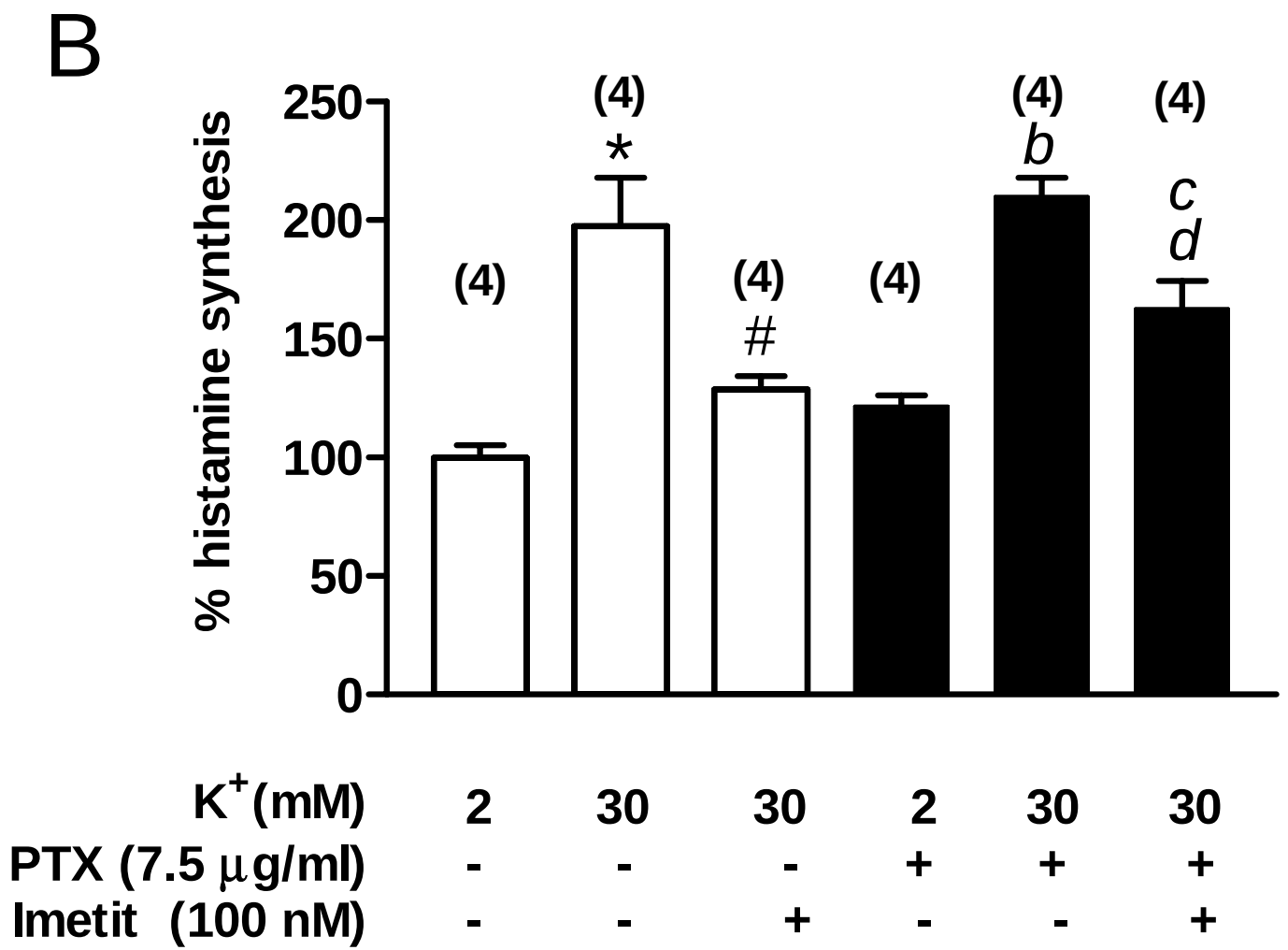


Figure 4B

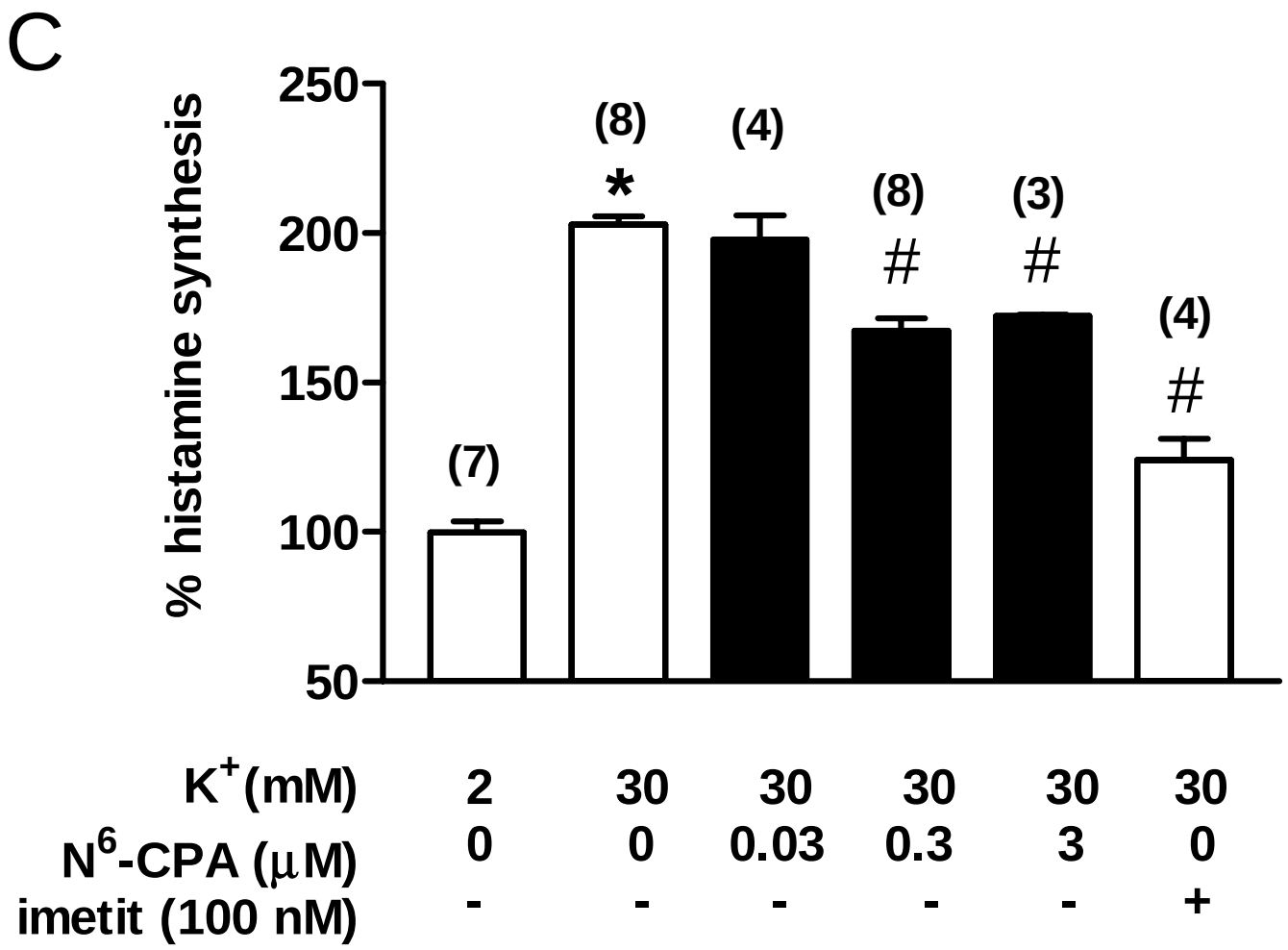


Figure 4C

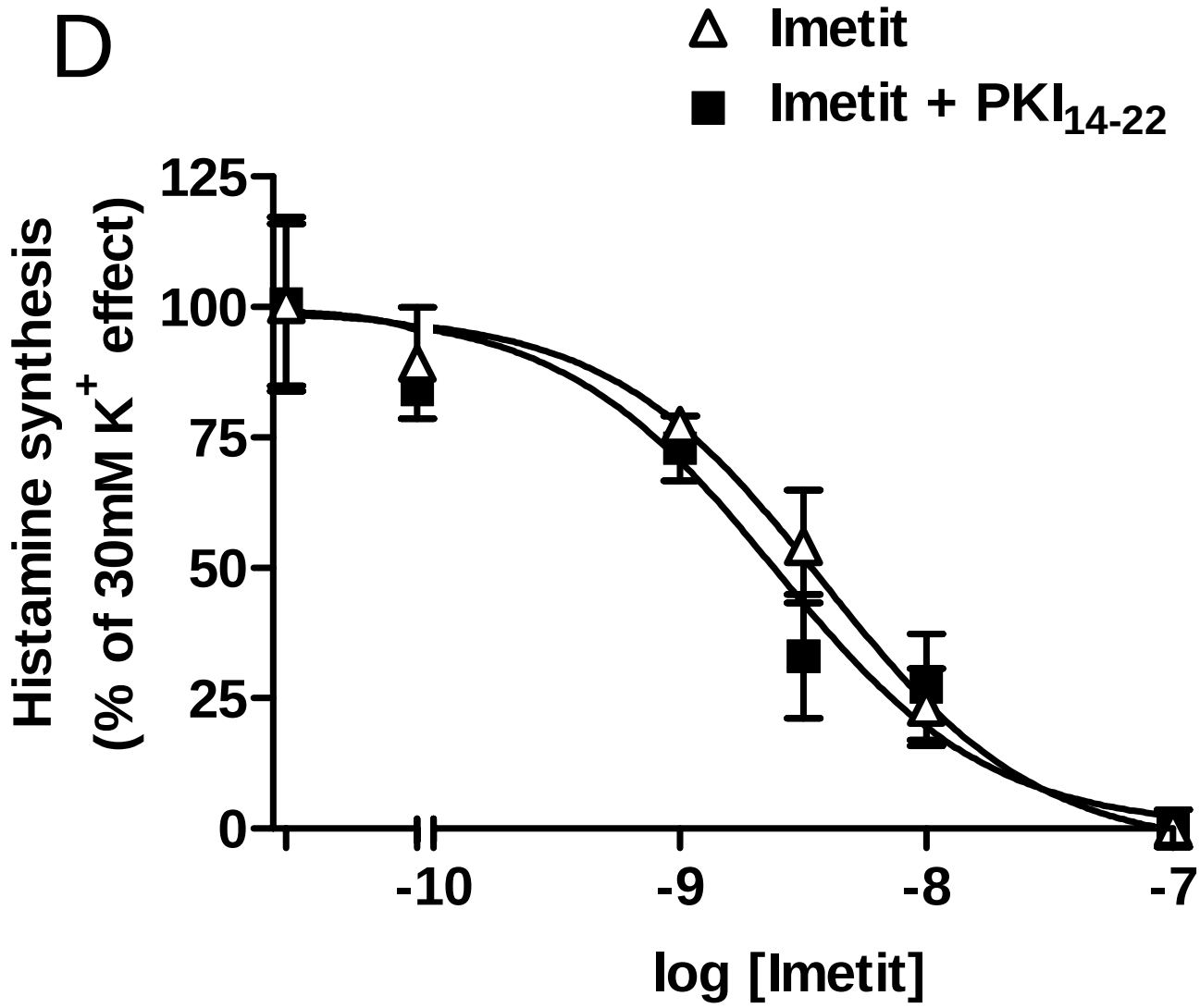


Figure 4D

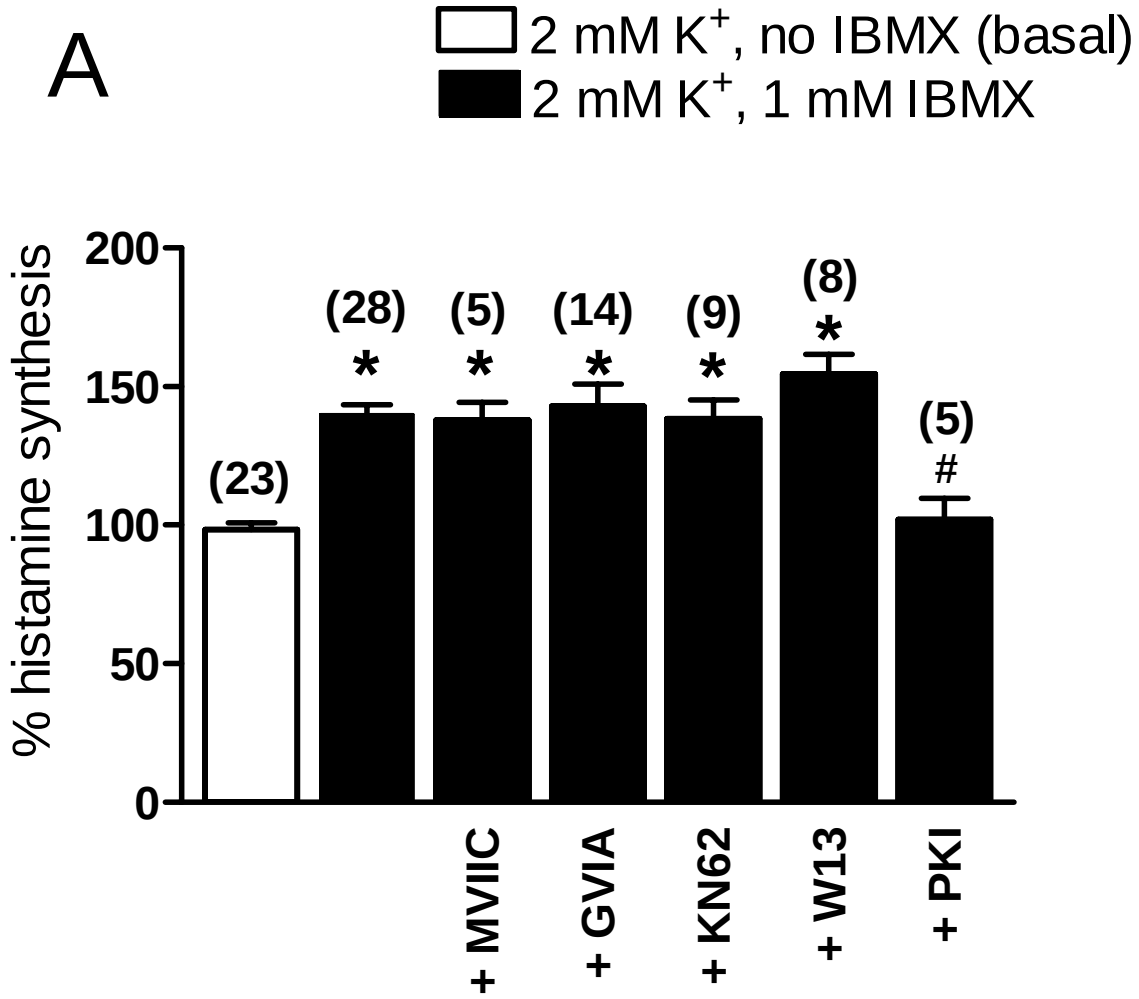


Figure 5A

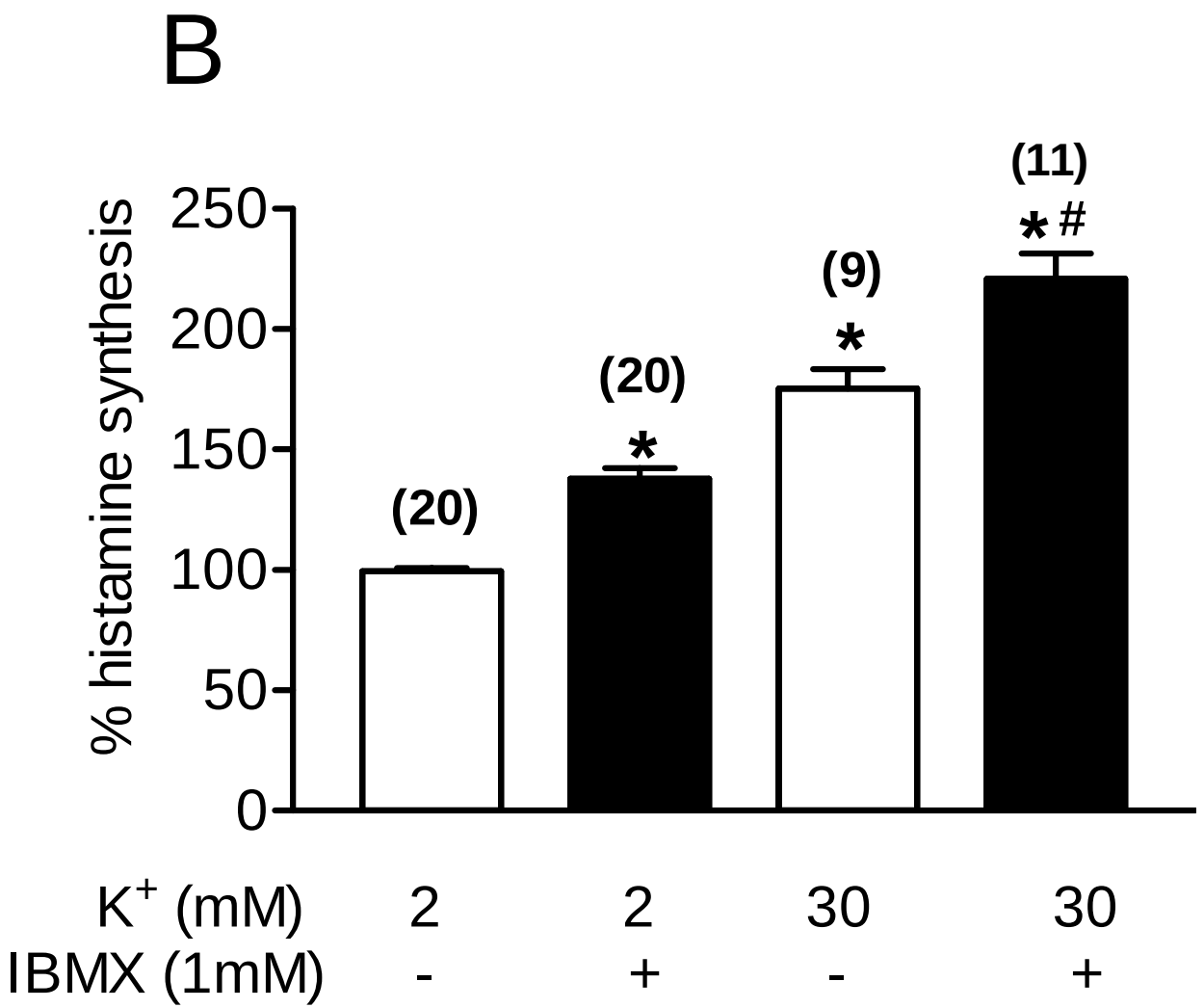


Figure 5B

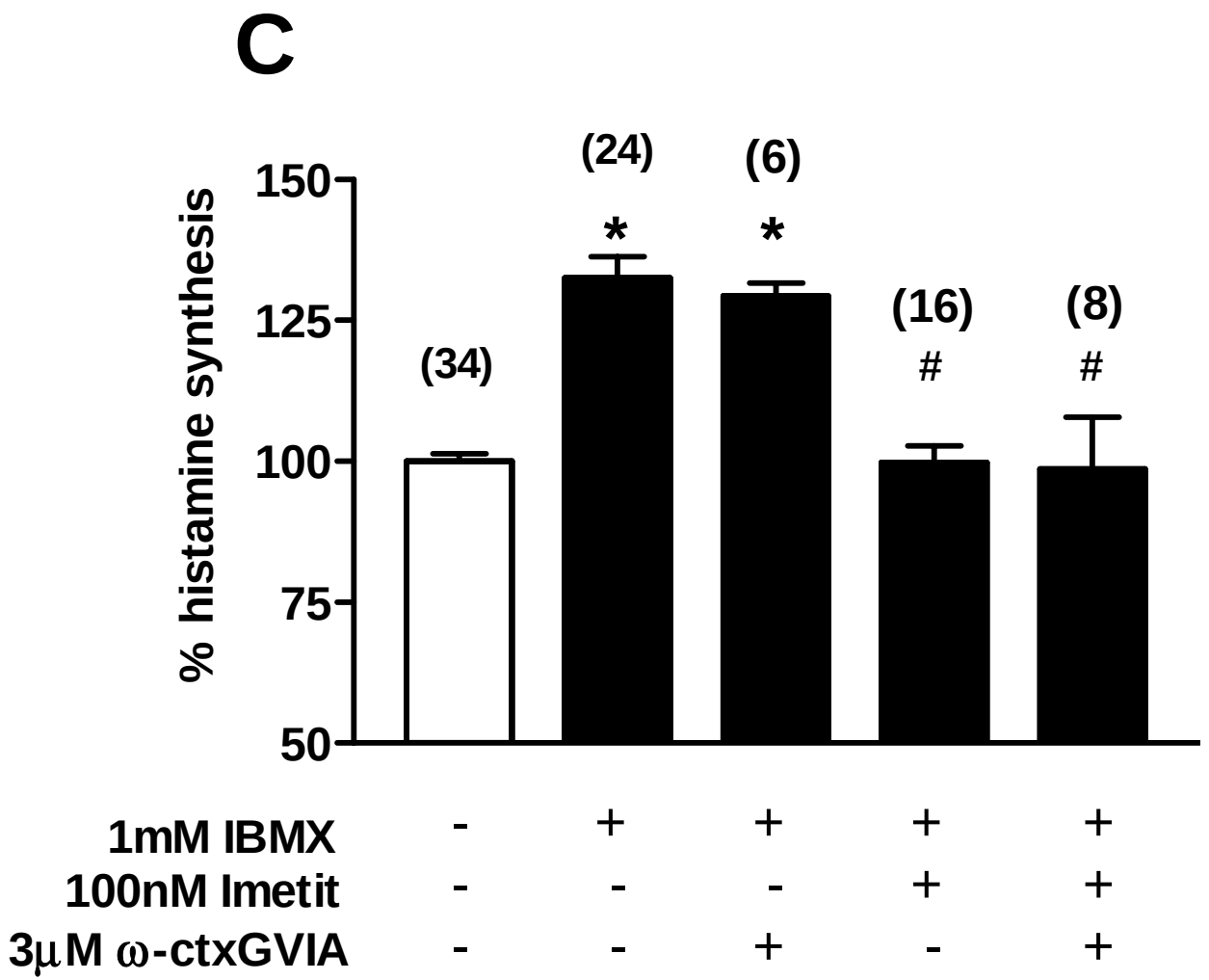
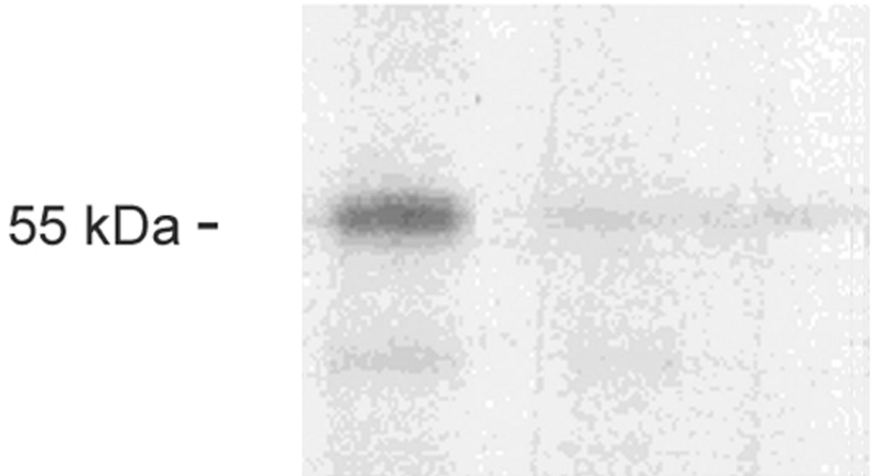


Figure 5C

A



HDC	+	-	+
PKA	+	+	-

Figure 6A

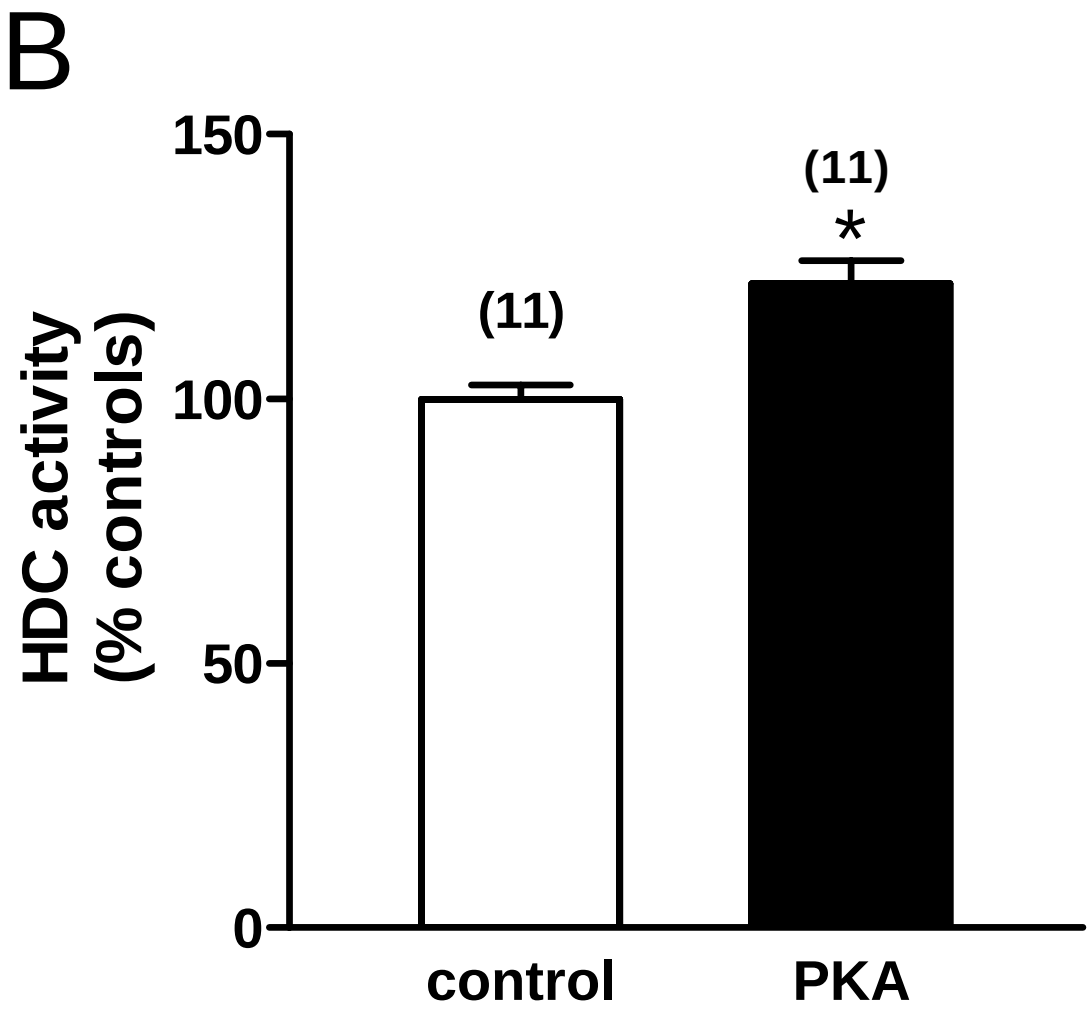


Figure 6B