CHAPTER 3:

“Involvement of cAMP and Ca$^{2+}$ on [$^3$H]-Histamine Release in rat brain cortical miniprisms”
Histamine $H_3$ receptor transduction through cAMP pathway modulates histamine synthesis but not histamine release

Jordi Gómez-Ramírez, Anna Torrent, Isaac Blanco* and Jordi Ortiz.

Department of Biochemistry and Molecular Biology, School of Medicine, Universitat Autònoma de Barcelona, E-08193, Bellaterra, Spain.

*Corresponding author: Dr. Isaac Blanco
Department of Biochemistry and Molecular Biology
School of Medicine
Universitat Autonoma de Barcelona
08193-Bellaterra (Spain)
Tel: (34) 93-581-16-25; Fax: (34) 93-581-15-73

Abbreviations:
$\omega$-CTX, $\omega$-conotoxin GVIA; AC, adenylate cyclase; $Ca^{2+}$, calcium; db-cAMP, dibutyryl-cAMP; dd-forskolin, dideoxy-forskolin; HA, histamine; HDC, histidine decarboxylase; HPLC, high performance liquid chromatography; IBMX, 3-isobutyl-1-methylxanthine; $K^+$, potassium; KRM, Krebs-Ringer-Bicarbonate medium; PKA, cAMP-dependent protein kinase; PKI$_{14-22}$, myristoylated cAMP-dependent protein kinase inhibitor 14-22 amide.
ABSTRACT

The regulation by cAMP pathway of histamine release and synthesis was investigated using rat brain cortical miniprisms. Histamine release and synthesis was simultaneously determined in each sample. The addition of db-cAMP or IBMX (1mM) to the medium increased histamine synthesis (+20% and +38%) but failed to produce any effect on histamine release. Potassium depolarization (30mM) increased both histamine synthesis (+113%) and release (+1363%). Db-cAMP and IBMX effects on histamine synthesis were additive to those of potassium. Notably, the presence of ω-conotoxin GVIA, which blocks N-type calcium channels, did not modify IBMX-stimulated histamine synthesis. On the other hand, inhibition of PKA did not modify depolarization-induced histamine release. As previously reported, the H₃ receptor agonist imetit reverted IBMX-stimulated histamine synthesis. The conotoxin did not impair imetit inhibition of IBMX-stimulated synthesis. However, ω-CTX could impair depolarization stimulation of both histamine synthesis and release indicating that extracellular calcium entry through N-type calcium channels is responsible of depolarization effects. These results demonstrate that H₃ receptor inhibition of adenylate cyclase and the effects of cAMP elevation on histamine synthesis are independent of extracellular calcium entry through N-type calcium channels.

Forskolin (100µM) increased both histamine release and synthesis either in basal (+260% and +58%) and depolarizing conditions (+1619% and +158%). Dideoxy-forskolin, a forskolin analog which does not activate adenylate cyclase, increased histamine release (+85%) and slightly stimulated synthesis (+21%) in basal conditions. ω-conotoxin GVIA totally reverted forskolin and dd-forskolin effects on histamine release. However, the conotoxin did not completely block forskolin effects on histamine synthesis. An inhibitor of PKA (PKI₄₋₂₂) strongly impaired forskolin-stimulated synthesis but not dideoxy-forskolin effects. These results suggest that forskolin has effects on adenylate cyclase activity and calcium entry. The latter can stimulate histamine release and synthesis.

In conclusion, these results indicate that cAMP levels do not modulate histamine release.
INTRODUCTION

Histamine (HA) is one of the aminergic neurotransmitters playing an important role in the regulation of several physiological and pathological processes. In the mammalian brain histamine is synthesized by a population of neurons whose cell bodies are restricted to the tuberomamillary nucleus of the posterior hypotalamus (Schwartz et al., 1991). These histaminergic neurons have been implicated in several brain functions (e.g. sleep/wakefulness, hormonal secretion, cardiovascular control, thermoregulation, food intake and memory formation).

Histamine H₃ receptors were first identified as presynaptic autoreceptors on brain histamine neurons although they are present also in many other cell types (Schwartz et al., 1991). H₃ autoreceptors control the depolarization-stimulated release of histamine (Arrang et al., 1983) as well as histamine synthesis (Arrang et al., 1987). Human and rat H₃ receptor cDNAs have also been cloned and functionally characterized (Lovenberg et al., 1999 and 2000) showing that the receptor belongs to the family of G-protein-coupled receptors. In these studies, H₃ receptor agonists were shown to decrease forskolin-stimulated cAMP in receptor-transfected cells. More recently, we reported that H₃ autoreceptors modulate histamine synthesis using the adenylate cyclase-cAMP protein kinase (AC-PKA) pathway in rat cerebral cortex miniprisms (Gomez-Ramirez et al., 2002). Although, these observations clearly link H₃ receptors to G-protein-coupled receptors, they do not exclude the possibility that additional signal transduction mechanisms are involved in H₃ autoreceptor-mediated events. It is well described that depolarization in presence of calcium increases histamine release and synthesis in brain miniprisms or synaptosomes. In these conditions, H₃ receptor ligands modulate both release and synthesis. Neurophysiological studies (Takeshita et al., 1998; Schlicker et al., 1994; Blandizzi et al., 2001) suggest that H₃-receptor modulation of depolarization induced-histamine release may be mediated by Ca²⁺ influx through voltage-dependent Ca²⁺ channels in histaminergic neurons. Since we have recently demonstrated that H₃ autoreceptors modulate histamine synthesis through cAMP pathway the possibility exists that this pathway modulates also histamine release. To confirm this hypothesis, we investigated the regulatory role of AC-PKA pathway on histamine release compared with its effect on histamine synthesis (Gomez-Ramirez et al., 2002). To this end, we slightly modified the procedure described by Arrang et al (1983) to determine histamine release and synthesis simultaneously in the same sample.
EXPERIMENTAL PROCEDURES

Materials:

Ring-labelled [2,5-\textsuperscript{3}H]L-histidine stocks (1 mCi, 50 Ci/mmol) were obtained from Amersham (Buckinghamshire, UK) and were purified by High Performance Liquid Chromatography (HPLC) before their use (Ortiz et al., 2000). Non-radiolabelled histidine, histamine, thioperamide maleate, imetit, octanesulfonic acid and trichloroacetic acid were purchased from Sigma / RBI (Steinheim, Germany). PKI\textsubscript{14-22}, db-cAMP and IBMX were obtained from Calbiochem / Merck KGaA (Darmstadt, Germany). Forskolin was supplied from Alomone-Labs (Jerusalem, Israel). Omega-Conotoxin GVIA was supplied by Tocris Cookson Ltd. (London, UK). All the other reagents were of the maximum purity available. IBMX, forskolin and dd-forskolin stocks were prepared in DMSO. Reverse-phase C18 HPLC column, 25 x 0.46 cm (Tracer Extrasil ODS-2, of 5 µm particle size) equipped with a 2 x 20 mm guard column (Upchurch) was purchased from Teknokroma (Barcelona, Spain). Amberlite IRA-900 (mesh 16-50) strong anion-exchange resin (Supelco) was also from Teknokroma. Microspin filter Ultrafree-MC tubes with low binding durapore membrane of 0.45 µm pore size were obtained from Millipore GmbH (Eschborn, Germany). OptiPhase "HiSafe"-3 liquid scintillation cocktail was purchased from Perkin-Elmer / Wallac (Turku, Finland).

Preparation and incubation of brain slices:

Male Sprague-Dawley rats of 200-250 g (Servei d’Estabulari, Universitat Autonoma de Barcelona, Spain) were killed by decapitation between 9-10h A.M. Brains were immediately placed into ice-cold modified Krebs Ringer bicarbonate medium (KRM) of the following composition (mM): 120 NaCl, 0.8 KCl, 2.6 CaCl\textsubscript{2}, 0.67 MgSO\textsubscript{4}, 1.2 KH\textsubscript{2}PO\textsubscript{4}, 27.5 NaHCO\textsubscript{3}, 10 glucose; pH 7.4. A McIlwain tissue chopper was used to obtain cerebral cortex miniprisms of 0.3 x 0.3 mm side. The miniprisms were suspended in KRM and washed three times with ice-cold KRM to remove debris of damaged cells. Aliquots of 100 µl from the settled slice suspension (usually containing 2-3 mg protein) were distributed (using a yellow pipette tip cut at 1 cm from the tip) into 2 ml polypropylene tubes. The tubes were preincubated for 25 min at 37ºC in a shaking water under O\textsubscript{2}/CO\textsubscript{2} (95:5) atmosphere. After preincubation, prepurified [\textsuperscript{3}H]-histidine (3.12 µCi, to make a final concentration of 0.25 µM) was added to all samples and the
tubes were incubated for 5 more minutes to allow for $[^3]H$-histidine uptake. Then, miniprisms were incubated for 30 minutes to synthesize $[^3]H$-histamine in a final volume of 250 µl. If depolarizing conditions were required, the buffer added to bring volume to 250 µl contained different concentrations of concentrated KCl. NaCl concentration was decreased proportionally to maintain the isotonicity.

Drugs tested (forskolin, dd-forskolin, db-cAMP, IBMX, PK14-22, ω-conotoxin GVIA and H3 receptor ligands) were added 15 min before the incubation period. Several concentrations of each compound were tested because of their difficulty to diffuse across the tissue. A difficult diffusion may explain why apparently high drug concentrations are needed to obtain intracellular effects relative to those needed in other preparations.

**Determination of $[^3]H$-histamine release:**

At the end of the incubation period, tubes were rapidly centrifuged at 700 x g for 15 sec at 4°C to settle the miniprisms. 150 µl of the supernatants were transferred to ice-cold tubes containing 35 µl of deproteinization mixture (25 µl of 10% trichloroacetic acid mixed with 10 µl of 10 mM histamine as internal standard). Blank samples were centrifuged just after the addition of labelled histidine. Blanks and samples were vortexed and then centrifuged at 14,000 x g for 10 min at 4°C. The supernatants were recovered and processed as follows in "Histamine purification by HPLC".

**Determination of $[^3]H$-histamine synthesis:**

As mentioned earlier, 150 µl were taken from the supernatants of settled miniprisms in order to determine histamine release. After that, the tubes containing settled miniprisms were placed on ice and added 35 µl of deproteinization mixture and 150 µl of KRM to compensate the volume removed previously. Then, the tubes were sonicated for 10-20 sec at 4°C using a Dynatech / Sonic Dismembrator. These samples were centrifuged at 14,000 x g for 10 min at 4°C and supernatants were recovered and used for determination of $[^3]H$-histamine as follows in "Histamine purification by HPLC". $[^3]H$-histamine synthesis was estimated by the sum of $[^3]H$-histamine remaining in these samples plus previously obtained $[^3]H$-histamine release.

**Protein quantification:**

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

**Histamine purification by HPLC:**

In short, $[^{3}H]$-histamine levels in each sample were determined by HPLC separation from $[^{3}H]$-histidine as described in Ortiz et al., 2000. The HPLC procedure separation was performed on a reverse-phase C18 column using ion-pair mobile phase. Non-radiolabelled histamine was used as internal standard as well as to monitor $[^{3}H]$-histamine elution from column.

Dpm obtained were corrected by recovery of the histamine internal standard, blank dpm values, specific activity of the labelled histidine, protein content of the tube containing the miniprisms and sample incubation time. To quantify the $[^{3}H]$-histamine synthesis, the sum of $[^{3}H]$-histamine release in each sample and $[^{3}H]$-histamine remaining in the tube containing the miniprisms was performed. IBMX interfered with consecutive HPLC injections due to its retention time of 50 min.

**Statistical analysis:**

Statistical significance of differences between values was evaluated by analysis of variance (ANOVA) followed by Dunnett and Bonferroni test for multiple group comparisons. Significance was established at $P<0.05$. 
RESULTS

Potassium depolarization stimulates histamine release and synthesis in a concentration and time-dependent manner.

To validate the conditions for determining histamine release in parallel with histamine synthesis, we studied the effect of depolarization with potassium in rat brain cortical miniprisms. As previously published (Arrang et al., 1983 and 1987B), potassium produced a concentration-dependent stimulation of histamine release (Fig.1A). At 60mM K⁺, it was obtained an increase of +1363.3 ± 34.2% versus 2mM K⁺. Similarly, potassium produced a concentration-dependent stimulation of histamine synthesis (Fig.1B) producing a stimulation of +113.9 ± 4.7% at 60mM K⁺ (compared with 2mM K⁺).

To assess whether K⁺ depolarization-stimulatory effect was linear over time, we studied the effects of 2mM and 30mM K⁺ at different incubation times. 30mM K⁺-stimulated histamine release (Fig.2A) increased linearly up to 30 minutes, rising to +712.0 ± 94.5% as compared with K⁺ 2mM samples incubated simultaneously. Basal release of [³H]-histamine in miniprisms incubated with K⁺ 2mM was also linear over time (Fig.2A). 30mM K⁺-stimulated histamine synthesis was also linear and it reached an increase of +84.7 ± 12.1% at 30 minutes compared with K⁺ 2mM samples incubated in the same conditions. These results demonstrate that it is possible to quantify simultaneously histamine synthesis and release in the same miniprisms depolarized with 30mM potassium during 30 minutes. Taking into account these data, we used these conditions in subsequent studies.

Db-cAMP and IBMX stimulate histamine synthesis but not histamine release.

Direct activation of PKA using db-cAMP, a membrane permeable cAMP analog, did not modify [³H]-histamine release (Fig.3A), although it increased histamine synthesis by +20.1 ± 3.0% compared with K⁺ 2mM (Fig.3B) as previously reported (Gomez-Ramirez et al., 2002). Similarly, IBMX, a phosphodiesterase inhibitor, did not alter [³H]-histamine release (Fig.3A). However, IBMX induced a rise of +38.1 ± 4.1% on [³H]-histamine synthesis versus K⁺ 2mM (Fig.3B) mediated by PKA (Gomez-Ramirez et al., 2002).
Figure 1. K⁺-depolarization stimulates histamine release and synthesis in a concentration-dependent manner. Results represent means ± SEM. Number of experiments is indicated in brackets above the columns. ** P<0.01 compared with K⁺ 2mM (Dunnett's test). 100% of [³H]-histamine release and synthesis in the presence of K⁺ 2mM and without drugs were 1.1 ± 0.1 and 15.3 ± 0.4 fmol HA x mg prot⁻¹ x h⁻¹, respectively.
Figure 2. 30mM potassium produces a time-dependent stimulation of histamine synthesis and release. Results represent means ± SEM. Each data point corresponds to three different replicates.
Figure 3. Db-cAMP and IBMX stimulate histamine synthesis but not histamine release in non depolarizing conditions. Db-cAMP and IBMX were added to the medium 15 min before the incubation period. Results are means ± SEM. Number of replicates is indicated in brackets above the columns. ** P<0.01 compared with K⁺ 2mM without drugs (Dunnett's test). 100% of histamine release and synthesis were 0.9 ± 0.1 and 13.8 ± 0.8 fmol HA x mg prot⁻¹ x h⁻¹, respectively.
Forskolin stimulates histamine release through non adenylate cyclase-mediated mechanisms.

We previously reported that forskolin stimulates histamine synthesis, although only part of this effect was blocked by a PKA inhibitor (Gomez-Ramirez et al., 2002). In this work, forskolin elicited an increase of histamine release and synthesis in a concentration-dependent manner. 200 µM forskolin increased [³H]-histamine release by $658.9 \pm 90.5\%$ (Fig.4A) and [³H]-histamine synthesis by $48.0 \pm 8.6\%$ (Fig.4B) as compared with K$^+$ 2mM. Dd-forskolin (at 200µM), a forskolin analog which does not activate adenylate cyclase (Seamon et al., 1983), elicited an increase of histamine release of $85.4 \pm 11.6\%$ (Fig.5A) and of histamine synthesis of $21.5 \pm 5.0\%$ (Fig.5B) versus K$^+$ 2mM.

To assess whether forskolin or dd-forskolin effects on release were dependent of calcium influx, we studied the effect of ω-conotoxin GVIA (ω-CTX), a selective N-type calcium channel blocker (Casali et al., 1997). ω-CTX totally reverted dd-forskolin stimulatory effects on release (Fig.6A) and synthesis (Fig.6B) as well as forskolin-stimulation of release (Fig.6A). However, the conotoxin only partially reverted forskolin-stimulatory effect on histamine synthesis (Fig.6B). A histamine synthesis increase of $19.8 \pm 3.3\%$ (compared with K$^+$ 2mM) remained, as expected due to the adenylate cyclase-mediated-forskolin effect (Gomez-Ramirez et al., 2002).

To assess whether the forskolin or dd-forskolin effects were mediated by cAMP production, we studied the effect of PKI$_{14-22}$, a potent and selective PKA inhibitor (Harris et al., 1997). PKI$_{14-22}$ did not impair histamine release elicited by forskolin or dd-forskolin (Fig.7A). However, it reverted forskolin-stimulated histamine synthesis by -73% (Fig.7B), as previously reported (Gomez-Ramirez et al., 2002). As expected, PKI$_{14-22}$ did not modify dd-forskolin-stimulated histamine synthesis.
Figure 4. Forskolin stimulates histamine release and synthesis in a concentration-dependent manner in non-depolarizing conditions. Forskolin was added to the medium 15 min before incubation period. Results represents means ± SEM. Number of replicates is indicated in brackets above the columns. ** P<0.01 compared with K⁺ 2mM without drugs (Dunnett’s test). 100% [³H]-histamine release and synthesis were 0.5 ± 0.1 and 12.9 ± 0.3 fmol HA x mg prot⁻¹ x h⁻¹, respectively.
Figure 5. dd-Forskolin stimulates histamine release and synthesis. dd-Forskolin was added to the medium 15 min before incubation period. Results represents means ± SEM. Number of replicates is indicated in brackets above the columns. ** P<0.01 and * P<0.05 compared with K+ 2mM without drugs (Dunnett's test). 100% [3H]-histamine release and synthesis were 0.8 ± 0.1 and 13.8 ± 0.7 fmol HA x mg prot⁻¹ x h⁻¹, respectively.
Figure 6: ω-CTX blocks forskolin-stimulated histamine release and partially reduces forskolin-stimulated histamine synthesis. ω-CTX was added to the medium 5 min before the addition of forskolin. Results are means ± SEM. Number of replicates is indicated in brackets above the columns. ** P<0.01 compared with K+ 2mM. ## P<0.01 compared with forskolin treatments. $$ P<0.01 compared with K+ 30mM (Bonferroni’s test). 100% of [3H]-histamine release and synthesis were 1.0 ± 0.1 and 14.3 ± 0.7 fmol HA x mg prot⁻¹ x h⁻¹, respectively.
Figure 7. PKI14-22 does not inhibit forskolin-stimulated release and dd-forskolin-stimulated histamine release and synthesis. However, as previously reported, it partially inhibits forskolin-stimulated histamine synthesis. PKI14-22 was added to the medium 5 min before forskolin or dd-forskolin addition. Results are means ± SEM. Number of replicates is indicated in brackets above the columns. ** P<0.01 and * P<0.05 compared with K+ 2mM without drugs. a P<0.01 compared with forskolin treatment (Bonferroni’s test). 100% of [3H]-histamine release and synthesis were 1.1 ± 0.1 and 14.2 ± 0.8 fmol HA x mg prot⁻¹ x h⁻¹, respectively.
Histamine synthesis can be stimulated by two independent mechanisms: AC-PKA and K+ depolarization.

In order to examine whether the effects of AC-PKA pathway activators and K⁺-depolarization were additive, we compared the effects of forskolin (100µM), IBMX (1mM) or db-cAMP (1mM) in 2mM or 30mM K⁺ on histamine release and synthesis. In K⁺ 2mM, forskolin produced and increase of synthesis of +57.9 ± 5.6%, IBMX of +38.1 ± 4.1% and db-cAMP of +20.1 ± 3.1%. K⁺ 30mM stimulated histamine synthesis by +75.2 ± 8.2%. In depolarizing conditions (30mM K⁺), the effects of AC-PKA activation were similar to those in K⁺ 2mM: IBMX and db-cAMP produced an increase on histamine synthesis of +45.7% and +43.9% compared with K⁺ 2mM, respectively (Fig.8B). However, they did not show any statistically significant effect on histamine release (Fig.8A). On the other hand, forskolin elicited an increase on histamine release of +492.3% (Fig.8A) and on synthesis of +82.9% (Fig.8B) in depolarizing conditions.

Blockade of Ca²⁺ channels does not impair the inhibition by imetit of IBMX-stimulated histamine synthesis in non depolarizing conditions.

We previously reported that the H₃ receptor agonist imetit could modulate IBMX-stimulated histamine synthesis (Gomez Ramirez et al., 2002). To study whether H₃ autoreceptors could require calcium influx in order to regulate histamine synthesis through AC-PKA, we studied the effect of ω-CTX in miniprisms treated with imetit plus IBMX. The blockade of Ca²⁺ channels failed to modify imetit-inhibition of IBMX-stimulated histamine synthesis in non depolarizing conditions (Fig.9B). Basal histamine release in non depolarizing conditions was not modified by imetit whilst depolarization-stimulated release was strongly inhibited (Fig.9A).
Figure 8. The effects of forskolin, db-cAMP and IBMX on histamine release and synthesis are additive with those of potassium depolarization. The drugs were added to the medium 15 min before the incubation period. Results are means ± SEM. Number of replicates is indicated in brackets above the columns. ** P<0.01 compared with K+ 2mM. ## P<0.01 compared with K+ 30mM samples (Bonferroni’s test). 100% of [3H]-histamine release and synthesis were 1.0± 0.1 and 17.5 ± 1.4 fmol HA x mg prot⁻¹ x h⁻¹, respectively. Note that db-cAMP or IBMX did not stimulate histamine release whereas forskolin stimulated both processes.
Figure 9. \(\omega\)-CTX does not modify imetit-inhibition of IBMX-stimulated histamine synthesis. \(\omega\)-CTX was added to the medium 5 min before the addition of IBMX and imetit. Results are means ± SEM. Number of replicates is indicated in brackets above the columns. ** P<0.01 compared with K+ 2mM without drugs. a P<0.01 compared with IBMX samples. b P<0.01 compared with K+ 30mM treatment (Bonferroni test). 100% of \([^3]H\)-histamine release and synthesis were 1.4± 0.1 and 13.9 ± 0.4 fmol HA x mg prot\(^{-1}\) x h\(^{-1}\), respectively.
DISCUSSION

We had previously shown that H₃ receptors modulate histamine synthesis through the adenylate cyclase-PKA pathway (Gomez-Ramirez et al., 2002). The aim of our present work was to investigate whether the AC-PKA pathway also contributes to H₃ receptor modulation of histamine release. Our results show that histamine release is not modified by increasing cAMP levels or PKA stimulation. In addition, PKA inhibition does not modify depolarization-induced histamine release. Thus, cAMP levels appear to be involved on the modulation of neurotransmitter synthesis, but not release. On the other hand, as expected, extracellular calcium entry is required for depolarization-induced histamine release. An association of H₃ receptors to voltage-sensitive calcium channels has also been described (Takeshita et al., 1998), which may constitute another transduction pathway of H₃ receptors. However, extracellular calcium entry is not required for H₃ receptor inhibition of IBMX-stimulated histamine synthesis, a cAMP mediated action. Taken together, these data suggest that histamine release is not cAMP-dependent as it is histamine synthesis. Histamine H₃ receptors should then modulate neurotransmitter release via calcium, but not cAMP. These two intracellular signals seem to have different roles in synaptic functions.

Histamine release can not be studied after incubation of slices with [³H]-histamine since no high-affinity histamine uptake appears to exist in neurons. Thus, we incubated the slices with [³H]-histidine as described by Arrang et al (1983) with some modifications in order to assess histamine synthesis and release within the same sample. This procedure allows us to compare the regulation by calcium or cAMP of histamine release with that of histamine synthesis. However, release processes are very fast for this reason the depolarization times used in the literature are very short. In contrast, histamine synthesis studies require longer incubations because synthesis is slower process rate-limited by histidine decarboxylase activity. Our data show that release was linear with time (up to 30 min) which indicates that released histamine in our conditions does not act on H₃ receptors to inhibit its own release. Release was dependent on the concentrations of KCl (Fig.1) as well as of the potassium channel blocker 4-aminopyridine (data not shown). We therefore concluded that [³H]-histamine release measured at 30 min was representative of depolarization mediated events.
A role of cAMP in neurotransmitter release has been previously sought by different authors, although results have been inconclusive in many respects. It is well known that some vesicular release proteins are phosphorylated by PKA (Turner et al., 1999; Hilfiker et al., 1999). However, PKA activity seems not to be rate-limiting on vesicle release, since direct injection of PKA catalytic subunits in the squid giant synapse does not stimulate release (Hilfiker et al., 2001). Similarly, cAMP elevations do not contribute to GABA release by cortical neurons (Zhao et al., 2001). Using microdialysis, Okada et al (2001) have shown that forskolin produces almost no stimulation of basal serotonin release in rat hippocampus. However, the same authors showed that forskolin does potentiate KCl-induced depolarization. IBMX or db-cAMP produce very modest increases of striatal dopamine release (West and Galloway, 1996). In the rat bladder cAMP seems to modulate noradrenaline, but not acetylcholine release (Somogyi et al., 1996). cAMP elevations do facilitate electrically evoked [3H]-noradrenaline release from chick sympathetic neurons, even though this second messenger does not participate in alpha-2 adrenergic inhibition of noradrenaline release (Boehm et al 1994). Taken together, these results would suggest that the involvement of cAMP in neurotransmitter release may be restricted to some systems or conditions. Other reports have shown that the cAMP role on release may be to modulate vesicle exocytosis to a given calcium signal. Positive regulatory effects of PKA on the timing of quantal release at the frog muscle endplate have been described (Bukhararova et al., 2002), as well as the vesicular cycling in cerebellar neurons (Chavis et al., 1998). This protein kinase facilitates the probability of vesicle exocytosis after a given calcium signal in hippocampal neurons (Trudeau et al., 1996), and participates in glutamate release in long-term potentiation (Weisskopf et al., 1994). PKA participates in presynaptic enhancement of synaptic transmission (Capogna et al., 1995) and modulation of glutamate release by metabotropic glutamate receptors (Evans et al., 2001) and beta-adrenergic receptors (Herrero and Sanchez-Prieto, 1996). Taken together, these results suggest that the AC-PKA may modulate synaptic efficacy. However, in agreement with our results, we have found no reports of cAMP levels mediating the modulation of neurotransmitter release by presynaptic autoreceptors.

How do \( H_3 \) receptors modulate histamine release? Voltage-sensitive calcium channels of the N- and P-type are modulated by \( H_3 \) receptors (Takeshita et al., 1998). The mechanisms of such inhibition is likely to be G protein beta-gamma subunits (Diverse-Pierluissi et al., 2000). \( H_3 \) receptor modulation of extracellular calcium entry is
also involved in acetylcholine (Blandizzi et al., 2001), glutamate (Molina-Hernandez et al., 2001) and noradrenaline release (Silver et al., 2002). In the present work we have shown that histamine release can be impaired by N-type calcium channel blocker ω-conotoxin. This result is in agreement with the view that H3 receptors modulate histamine release through calcium channel modulation.

Histamine synthesis can also be stimulated by depolarization-induced calcium entry. This effect can be impaired by ω-conotoxin GVIA, which shows that the effect is mediated by calcium entry through N-type channels. We are currently investigating what mechanisms may be involved in the effects of calcium on histamine synthesis. PKA must not be involved in this mechanism, since this protein kinase inhibition does not decrease depolarization-induced stimulation of synthesis. Moreover, the increases of histamine synthesis by depolarization and PKA activation are independent of each other, as shown by the fact that IBMX or db-cAMP effects are not modified by depolarization. The effects of PKA activation are additive to those of depolarization, not synergistic. Furthermore, IBMX effects are not modified by ω-CTX, which rules out the possibility that IBMX stimulation of histamine synthesis could be mediated by PKA phosphorylation of calcium channels. Thus, there should be at least two pathways leading to increases of histamine synthesis, one mediated by calcium and the second by cAMP, and so far they appear to be independent of each other.

Forskolin is generally known as an adenylate cyclase activator. In an apparent disagreement with our conclusions, forskolin increased both histamine release and synthesis. Subsequent studies confirmed that this diterpene compound produced a dual effect: a stimulation of histamine release and synthesis through a calcium influx-dependent process and an increase of histamine synthesis through AC-PKA pathway. It is well known that forskolin has effects not mediated by adenylate cyclase such as the modulation of ion channels (Laurenza et al., 1989). Thus we assessed whether part of forskolin effects were not associated to adenylate cyclase activation: a) dideoxy-forskolin, a forskolin analog which does not activate AC, stimulated both release and synthesis, b) ω-conotoxin GVIA, a N-type calcium channel blocker, totally blocked forskolin-stimulated histamine release whilst an increase of histamine synthesis remained and c) PKI14-22, a selective PKA inhibitor, partially reverted forskolin stimulation of synthesis whereas it did not modify forskolin-stimulated release as well as
dd-forskolin-induced effects. These results demonstrate that forskolin effects on release are not mediated by AC, but they are mediated by calcium entry instead.

Given that the AC-PKA pathway does not seem to modulate histamine release, it can be assumed that the H₃ autoreceptor would not regulate histamine release through this cascade. As expected, ω-CTX could not block the inhibition by imetit of IBMX-induced-histamine synthesis. This would clearly confirm that histamine H₃ receptors use the AC-PKA pathway to modulate histamine synthesis. However, release would be dependent on extracellular calcium entry and modulated by yet unidentified intracellular mechanisms. In conclusion, our data demonstrate that cAMP does not mediate H₃ receptor modulation of histamine release.

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IV. CONCLUSIONS
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In this work, we have developed a procedure to purify by HPLC $[^3\text{H}]-\text{histamine}$ synthesized from radiolabelled histidine. Subsequently, this method has been applied to the study of the $H_3$ autoreceptors signal transduction pathways involved in the regulation of histamine synthesis and release in rat brain cortical miniprisms.

The conclusions obtained from the thesis are the following:

1) The radioisotopic method developed allows simpler, more sensitive and accurate quantification of $[^3\text{H}]-\text{histamine}$ compared to other previous methods. This procedure has been used to determine histidine decarboxylase activity in homogenates as well as $[^3\text{H}]-\text{histamine}$ synthesis in brain miniprisms. This methodology has been further validated by reproducing previously reported results such as the enzyme distribution in different rat brain areas or subcellular fractions as well as $H_3$ receptors functionality in histamine release and synthesis in brain miniprisms.

2) $H_3$ autoreceptors modulate $[^3\text{H}]-\text{histamine}$ synthesis through AC-PKA pathway. Based on our data we can not conclude whether PKA activates histidine decarboxylase through direct phosphorylation. Additional studies will be necessary to confirm this hypothesis.

3) PKA activation or inhibition do not modify histamine release. Our results suggest that AC-PKA cascade is not involved on $H_3$ autoreceptors modulation of histamine release.

4) Depolarization-elicited increase of $[^3\text{H}]-\text{histamine}$ release and synthesis is mediated by calcium influx through N-type calcium channels. Given that $H_3$ autoreceptors modulation of calcium entry through voltage-gated calcium channels has been described, it is likely that $H_3$ receptor modulation of histamine release is mediated by this second messenger. Now, it will be of interest to investigate which calcium dependent proteins are involved in the control of histamine synthesis and release in histaminergic neurons.

5) Histamine synthesis can be increased by AC-PKA activation and calcium entry in an independent manner. Taking into account that $H_3$ receptors activate simultaneously
both pathways, it could be possible that H₃ receptors act on both pathways to control histamine synthesis.

**Figure 1.** Schematic illustration of a histaminergic nerve ending showing the H₃ autoreceptor modulation of histamine release and synthesis. Compounds used to study the involvement of calcium and cAMP on these processes are indicated. Stimulatory and inhibitory effects are shown with (+) and (-), respectively. Indirect-mediated effects by several compounds are indicated in dashed lines. Abbreviations: AC, adenylate cyclase; PKA, protein kinase A; FOR, forskolin; HDC, histidine decarboxylase, HA, histamine, HIS, histidine; PDE, phosphodiesterase; PKI₁₄₋₂₂, PKA inhibitor; ω-CTX, ω-conotoxin GVIA; VACC, voltage-activated calcium channel.
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