Modulatory effects of histamine on cat carotid body chemoreception

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ABSTRACT

Histamine has been proposed to be an excitatory transmitter between the carotid body (CB) chemoreceptor (glomus) cells and petrosal ganglion (PG) neurons. The histamine biosynthetic pathway, its storage and release, as well as the presence of histamine H1, H2 and H3 receptors have been found in the CB. However, there is only indirect evidence showing the presence of histamine in glomus cells, or weather its application produces chemosensory excitation. Thus, we studied the histamine immunocytochemical localization in the cat CB, and the effects of histamine, and H1, H2 and H3 receptor blockers on carotid sinus nerve (CSN) discharge, using CB and PG preparations *in vitro*. We found histamine immunoreactivity in dense-cored vesicles of glomus cells. Histamine induced dose-dependent increases in CSN discharge in the CB, but not in the PG. The H1-antagonist pyrilamine reduced the CB responses induced by histamine, the H2-antagonists cimetidine and ranitidine had no effect, while the H3-antagonist thioperamide enhanced histamine-induced responses. Present data suggests that histamine plays an excitatory modulatory role in the generation of cat CB chemosensory activity.

1. Introduction

The carotid body (CB) is the main arterial chemoreceptor, which senses the arterial blood levels of P_{O_2}, P_{CO_2} and pH, contributing to the ventilatory and cardiovascular homeostasis (González et al., 1994). The glomoid is the basic morpho-functional unit of the CB, consisting of clusters of chemoreceptor (glomus or type I) cells synaptically apposed to the nerve terminals of petrosal ganglion (PG) neurons, all engulfed by sustentacular (type II) cell processes. The current model of CB chemoreception states that natural stimuli, like hypoxia and hypercapnia, depolarize glomus cells, increasing the intracellular [Ca^{2+}] and releasing several molecules, which acting on specific postsynaptic receptors located in the nerve terminals of PG neurons, increase the chemosensory frequency of discharges (Iturriaga and Alcayaga, 2004; Nurse, 2005; Prabhakar, 2006; Iturriaga et al., 2007). In response to natural stimuli, glomus cells release molecules such as acetylcholine (ACh), adenosine nucleotides, dopamine and peptides, which may act as excitatory transmitters or modulators between glomus cells and PG nerve terminals (González et al., 1994; Iturriaga and Alcayaga, 2004; Iturriaga et al., 2007).

Recently, Koerner et al. (2004) presented evidence suggesting that histamine, a central nervous system modulator of ventilation (Dutschmann et al., 2003), may act as an excitatory transmitter in the rat CB. Using radioimmunoassay they found that incubation of the isolated rat CB with a hypoxic solution increases the histamine content in the supernatant. They also reported the presence of the histamine biosynthetic enzyme histidine decarboxylase and the vesicular monoamine transporters VMAT1 and VMAT2 in the CB, as well as the expression of mRNAs of H1, H2 and H3 histamine receptors in the CB (Koerner et al., 2004). More recently, Lazarov et al. (2006) found positive immunoreactivity for H1 and H3 histamine receptors in the rat glomus cells and PG neurons. Thus, histamine meets some of the criteria to be considered a transmitter in the CB (Eyzaguirre and Zapata, 1984). However, it is not known if histamine is present in glomus cells associated to releasing structures, or if is restricted to mast cells in the CB (Heath et al., 1987). Another problem to consider histamine as an excitatory transmitter is the reported lack of excitatory effects on CB chemosensory discharges. Landgren et al. (1954) found that histamine applied through the carotid artery of cats *in situ* had no effect on CB chemosensory discharges. Indeed, intracarotid injections of histamine hydrochloride (2–50 μg) did not increase chemosensory discharges in normoxic breathing cats (Landgren et al., 1954). Nevertheless, it is well known that histamine produces marked vascular effects in cats (Champion and Kadowitz, 1997), which may affect the CB chemosensory

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process. Lazarov et al. (2006) found that the application of H1 and H3 receptor specific agonists to the rat CB caused a mild increased of phrenic nerve activity in a working heart–brainstem preparation, suggesting that histamine may excite the CB, but not direct proof of CB excitation induced by histamine was provided in this study. Accordingly, to test if histamine may act as an excitatory transmitter between glomus cells and PG neurons, we studied the immunolocalization of histamine in the cat CB, and the effects of exogenous histamine applications on CB chemosensory discharges using *in vitro* perfused or superfused CB preparations that had been extensively used to measure the chemoreceptor function and allows the separation of direct, vascular and systemic effects (Alcayaga et al., 1988; Iturriaga et al., 1991; Rey and Iturriaga, 2004), and in an isolated preparation of the PG (Alcayaga et al., 1998). The PG preparation allows the recording of antidromic discharges from the carotid sinus nerve (CSN), evoked by the application of putative transmitters and drugs to the surface of the PG neurons (Iturriaga et al., 2007).

2. Methods

2.1. Animals

Experiments were performed on 22 male adult cats (1.5–4.7 kg) anesthetized with sodium pentobarbitone (40 mg kg^{-1}, i.p.), followed by additional doses (12 mg, i.v.) to maintain a level of surgical anesthesia. The experimental protocol was approved by the Bio Ethical Committees of the Facultad de Ciencias Biológicas of the Pontificia Universidad Católica de Chile and the Facultad de Ciencias of the Universidad de Chile, and was performed according to the Guiding Principles for the Care and Use of Animals of the American Physiological Society.

2.2. Immunohistochemical and cytochemical studies

To study the presence of histamine immunoreactivity (HA-ir) in the CB, 8 CBs were removed from 5 anesthetized cats, fixed by immersion in 2.5% glutaraldehyde at 4 °C in 10 mM phosphate buffer, pH 7.2, for 12 h at 4 °C, dehydrated in graded ethanol series follow by xylol and then included in paraffin. The CBs were cut into 5 μm-thick sections, mounted on silanized slides, and the tissue slices were deparaffinized in xylol and rehydrated in graded ethanol series. The slices were treated for 10 min with 1% H_2O_2 to inhibit endogenous peroxidase, 2 mg/ml $NaBH_4$ for 10 min, and incubated for 48 h at 4 °C in a humidity chamber with a monoclonal antibody against histamine (1:100, Mab AHA-2, Fujiwara et al., 1997, 1999). Fujiwara et al. (1997) demonstrated that the histamine antibody used in this study is specific to histamine with an enzyme-linked immunoabsorbent assay (ELISA) binding test, simulating the immunohistochemistry of tissue sections, and the antibody does not react with any of the other amino acids tested or peptides with N-terminal histidine tested. However, we examine the specificity of the histamine antibody in the cat CB performing preabsorption controls. The Mab AHA-2 antibody was incubated with histamine–glutaraldehyde–bovine serum albumin (HA–GA–BSA, 2 μg/ml conjugate concentration) for 10 min at room temperature and with histidine–glutaraldehyde–bovine serum albumin conjugate (histidine–GA–BSA, 100 μg/ml conjugate concentration) for 10 min at room temperature. The HA–GA–BSA conjugate and the histidine–GA–BSA conjugate were prepared as previously described (Fujiwara et al., 1997). The samples were then incubated for 48 h at 4 °C in a humidity chamber with the mixture of the Mab AHA-2 preabsorbed with HA–GA–BSA conjugate or with the Mab AHA-2 with Histidine–GA–BSA conjugate. Finally, the slices were stained with a biotin-streptavidin peroxidase kit (UltraVision Detection System, LabVision, USA), and then counterstained with Harris' hematoxylin and permanently mounted. Photomicrographs were taken with a Nikon DXM1200 CCD camera coupled to a Nikon Eclipse E400 microscope (Nikon Corp., Japan). For immunofluorescence studies, CB slides were exposed to UV light for 30 min (Neumann and Gabel, 2002) and treated with $NaBH_4$ 0.1% to quench the tissue autofluorescence (Tagliaferro et al., 1997). The slices were then incubated for 48 h at 4 °C with a mixture of Mab AHA-2 and rabbit anti-tyrosine hydroxylase antibody (1:100, AB152, Chemicon, USA). Tyrosine hydroxylase (TH) is considered a marker of glomus cells (González et al., 1994). The CB slides were sequentially incubated with fluorescent secondary antibodies anti-mouse IgG (AlexaFluor594, Molecular Probes, USA) and anti-rabbit IgG (AlexaFluor488, Molecular Probes, USA). Finally, the preparations were mounted and observed in an Olympus confocal laser scanning microscope.

The immunocytochemical detection of HA-ir was performed in 5 CBs fixed overnight by immersion in cold 2.5% glutaraldehyde at 4° in 10 mM phosphate buffer, pH 7.2. The CBs were cut into 50 μm-thick sections in a microslicer and samples were treated with 1% H_2O_2 for 10 min, rinsed twice in phosphate-buffered saline (PBS, Sigma, USA), treated with 2 mg/ml $NaBH_4$ for 10 min, rinsed 3 times in TRIS-buffered saline (Sigma, USA) and incubated for 1 h in protein block serum free solution (Dako, Denmark). Samples were then incubated 48 h at 4 °C with Mab AHA-2 (1:100). Slices were stained with a biotin-streptavidin peroxidase kit (Sigma, USA) and revealed with 10 mg 3,3-diaminobenzidine tetrachloride (Sigma, USA) in PBS supplemented with 10 μl H_2O_2 30% for 10–15 min at 35 °C. Samples were treated with 1% osmium tetroxide in cacodylate buffer (pH 7.2) for 30 min and then with 2% aqueous uranyl acetate, dehydrated in ethanol, and embedded in an epoxy resin. Areas to be examined by electron microscopy were selected and ultrathin sections were obtained with an ultramicrotome, placed on 300-mesh copper electron microscopy grids. For the immunocytochemical detection of HA-ir, the samples were not counterstained. On the contrary, for normally ultrastructural studies the section were counterstained with uranyl acetate and lead citrate, and examined using a transmission electron microscope (Phillips Tecnai 12 Bio Twin, Netherlands).

2.3. In vitro recording of carotid sinus nerve discharges

The effects of 0.5–2000 μg histamine hydrochloride on CB chemosensory discharges was assessed using *in vitro* perfused or superfused CB preparations, which allow the isolation of vascular effects. The superfused CB preparation has been used to study chemoreceptor function without the interference of vascular control (Alcayaga et al., 1988; Eyzaguirre and Koyano, 1965). The CB with the carotid sinus nerve (CSN) was excised from cats and superfused *in vitro* with modified Tyrode solution equilibrated with 100% O_2, pH 7.40 at 38.5 ± 0.5 °C (Alcayaga et al., 1988). To study the participation of the blood vessels in the CB chemosensory process, we used an arterially perfused CB preparation, which conserve its functional vascularization (Belmonte and Eyzaguirre, 1974; Iturriaga et al., 1991). Briefly, the carotid bifurcation including the CB and the CSN, was excised from cats and perfused *in vitro* by gravity at constant pressure of 80 Torr with Tyrode's solution equilibrated with 20% O_2 and 5% CO_2 and simultaneously superfused with Tyrode's solution equilibrated with 95% N_2 and 5% CO_2 at 38.5 ± 0.5 °C and pH 7.40, as previously described (Iturriaga et al., 1991). To assess the effect of histamine hydrochloride (0.5–2000 μg) on the soma of PG neurons, the PG with the CSN was removed from cats and placed in a chamber as previously described (Alcayaga et al., 1998). The isolated PG was superfused with Hank's balanced solution containing

5 mM HEPES at 38.0 ± 0.5 °C, pH 7.42, and equilibrated with 21% O_2. To record the nerve activity in CB and PG preparations, the CSN was placed on a pair of platinum electrodes and lifted into mineral oil. The neural signals were preamplified and amplified, filtered (10 Hz to 1 kHz; notch filter, 50 Hz), and fed to an electronic amplitude discriminator, which allowed the selection of action potentials of a given amplitude above the noise to be counted with a frequency meter to measure the frequency of CSN discharges (f_{CSN}), expressed in Hz. The f_{CSN} signal was digitized with an analog-digital board (DIGITADA 1200, Axon Instruments, USA) and acquired in real-time using commercial (Axoscope, Axon Instruments, USA) or custom made programs.

Histamine hydrochloride, acetylcholine hydrochloride and nicotine bitartrate were applied to the superfused CB and isolated PG, under constant flow (1–1.5 ml/min), in 10 μl boluses with a microdispenser, whose tip was placed about 1 mm from the surface of the organs. In the perfused CB, histamine hydrochloride was applied in 200 μl boluses into the arterial line. The maximal responses of the CB and PG preparations were tested using acetylcholine (ACh) hydrochloride or nicotine bitartrate. Perfused

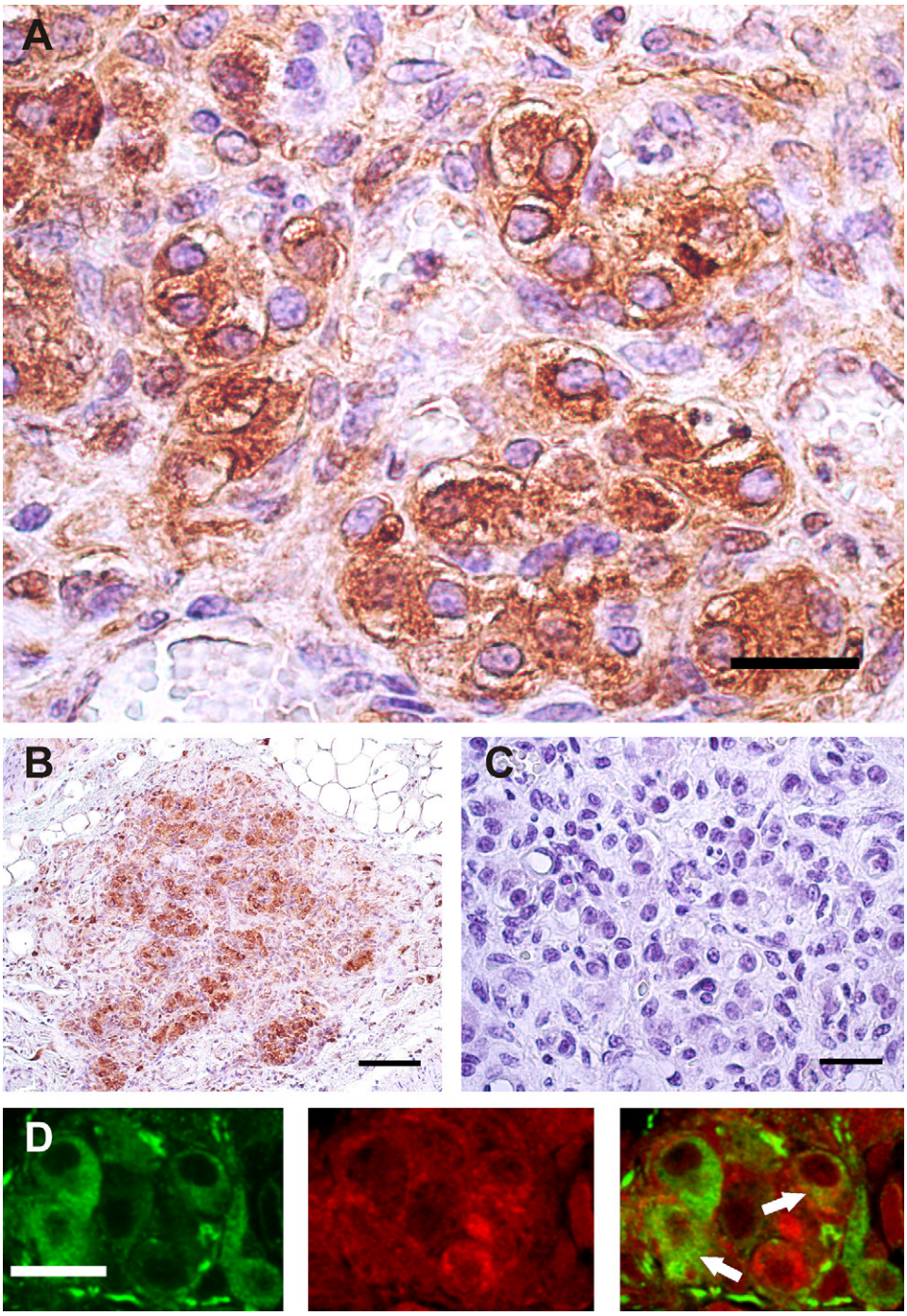

Fig. 1. Immunolocalization of histamine in the cat CB. (A) High magnification photomicrograph of the CB showing HA-ir in glomus cells clusters; bar, 20 μm. (B) Low magnification, bar, 100 μm. (C) No staining was found when the primary antibody was omitted; bar, 40 μm. (D) Double-labeling immunofluorescence of the CB for histamine (red fluorescence) and the glomus cell marker TH (green fluorescence). Arrows indicate double labeling immunofluorescent cells. Bar, 10 μm.

and superfused CB preparations were also excited with hypoxic stimulation ($P_{O_2} \sim 25$ Torr). Histamine receptor antagonists were applied at constant concentrations through the perfusion or superfusion medium to the CB. We used the H1 blocker pyrilamine (500 nM to 100 μM), the H2 blockers cimetidine (50 μM) and ranitidine (100 μM), and the H3 blocker thioperamide (30 μM). Doses corresponded to the salts. All drugs were obtained from Sigma (USA). During the application of 100 μM pyrilamine, the functional integrity of nerve conduction in the CB superfused preparation was evaluated by applying brief electrical pulses of 50 ms to the CB to record the compound action potentials in the CSN (Alcayaga et al., 1998).

2.4. Data analysis

Data was expressed as mean ± S.E.M. The change in frequency discharge (Δf_{CSN}) was calculated as the difference between the maximal frequency achieved during a single response and the mean basal activity, computed in a 30-s interval prior to an evoked response. To compare data from different experiments, f_{CSN} was expressed as a percentage of the maximal response attained with ACh or nicotine bitartrate ($\%f_{max}$) or as a $\Delta f_{CSN}/\Delta f_{max}$. To assess the effect of histamine on chemosensory discharges, the data was fitted to the following logistic expression: $\Delta f_{CSN} = 1/(1 + \{ED_{50}/D\}^S)$, where D = dose, ED_{50} = mean effective dose, S = Hill slope factor determining the steepness of each curve. The correlation coefficients for all adjusted curves were >0.90 ($p < 0.01$) for all conditions studied.

3. Results

3.1. Cellular localization of histamine in the cat carotid body

The HA-ir staining was present in the cat CB in clusters of cells that had been described as glomus cells in classical light microscope studies (Fig. 1A and B). Indeed, most of the positive HA-ir staining was found in clusters of round to ovoid cells, with a diameter of about 10 μm and a prominent nucleus (Fig. 1A). However, not all the glomus cell clusters presented HA-ir (Fig. 1A and B). The HA-ir staining was also detected in mast cells, mainly located in the interlobular connective tissues and around blood vessels (not shown). Mast cells were not associated with glomus cells. Negative controls, omitting the primary anti-histamine antibody were consistently devoid of immunoreactive staining (Fig. 1C).

3.2. Colocalization of histamine and tyrosine hydroxylase in carotid body glomus cells

To further characterize the cell type that presented HA-ir in the CB, we studied the immunolocalization of histamine and tyrosine hydroxylase (TH), a marker of glomus cells in 3 CBs using confocal microscopy. Colocalization of HA-ir and TH-ir in glomus cells was shown by double staining with secondary fluorescent antibodies. Fig. 1D shows the double-labeling immunofluorescence of a cluster of glomus cells for the glomus cell marker TH (green fluorescence) and HA-ir (red fluorescence), indicating that some glomus cells contain histamine (yellow fluorescence).

3.3. Preabsortion controls

No HA-ir was observed in the cat CB when the histamine antibody was preabsorbed with the HA–GA–BSA conjugate (Fig. 2A). On the contrary, HA-ir was present in glomus cells clusters

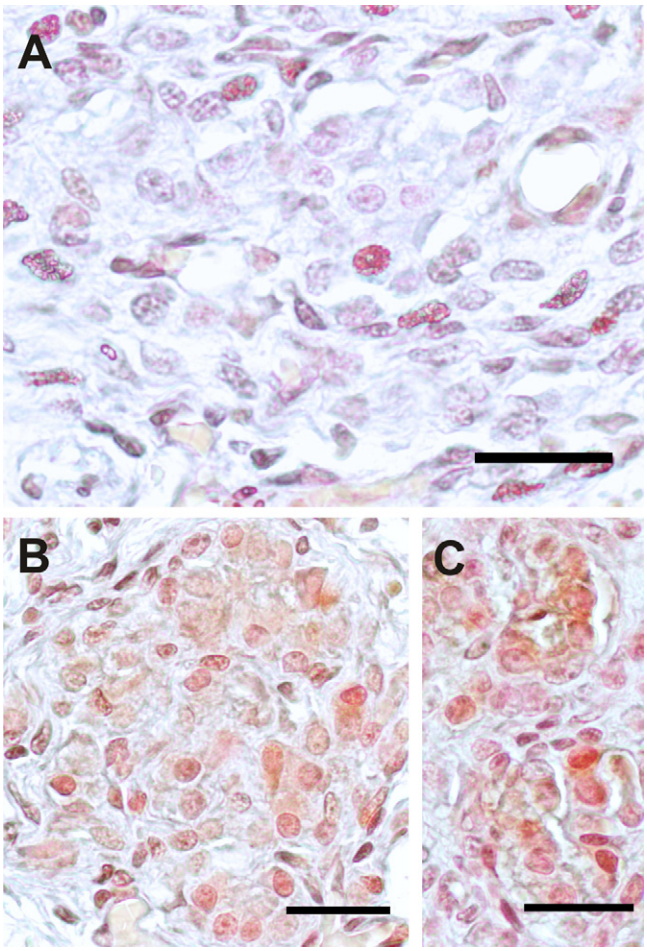

Fig. 2. Preabsorption controls of the histamine antibody with conjugates of histamine–glutaraldehyde (GA)–bovine serum albumin (BSA) and histidine–GA–BSA in the cat CB. High magnification photomicrograph of the same CB showing: (A) no HA-ir by preabsorption of the histamine antibody with HA-GA-HSA (2 μg/ml). (B) HA-ir in glomus cells clusters stained by the histamine antibody. (C) HA-ir in glomus cells clusters stained by the histamine antibody preabsorbed with histidine–GA–BSA (100 μg/ml). Bar, 20 μm.

in the CB when the histamine antibody was preabsorbed with the histidine–GA–BSA conjugate. Even large concentrations of histidine–GA–BSA conjugate (up to 100 μg/ml) could not eliminate the HA-ir (Fig. 2C). The HA-ir was confined to the same cell type (glomus cells) when the antibody was preabsorbed with histidine–GA–BSA or when the samples where incubated with the histamine antibody alone (Fig. 2B).

3.4. Ultrastructural studies of immunolocalization of histamine in glomus cells

The ultrastructural study showed that HA-ir was present in membrane-bounded electron-opaque granules in the cytoplasm of glomus cells (Fig. 3A and B). The presence of dark dense-cored granules in electron microscope sections (Fig. 3B, inset normal ultrastructure of glomus cells counterstained with uranyl acetate) is a distinctive characteristic that allows classifying these cell type as glomus cells. Fig. 3B shows that HA-ir was associated with structures that resemble the dark dense-cored vesicles containing dopamine in glomus cells (González et al., 1994). No HA-ir staining was found associated with vesicles when the primary antibody was omitted (data not shown).

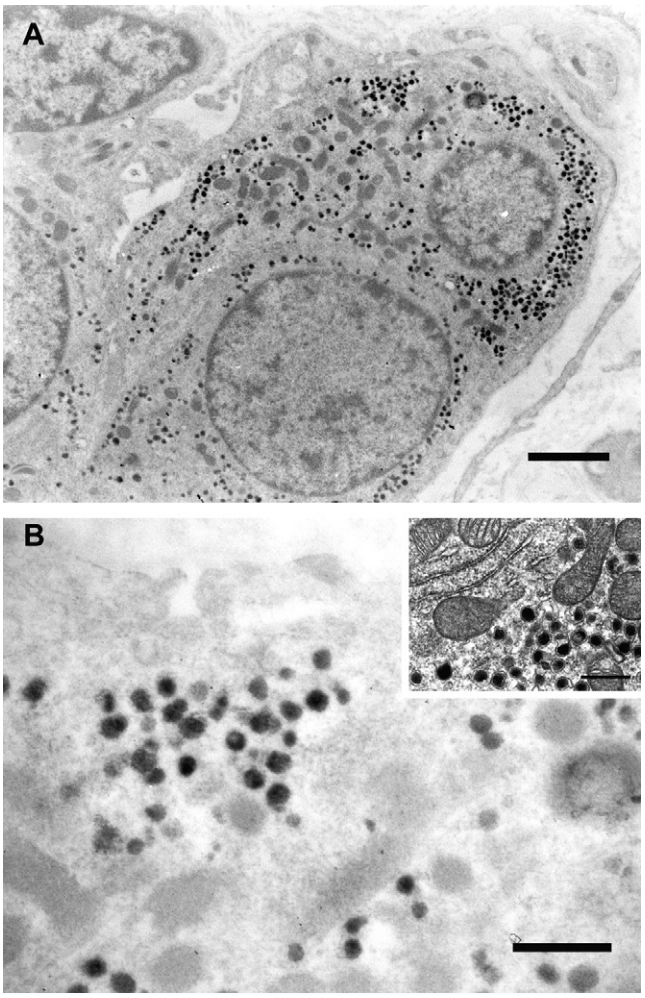

Fig. 3. Electron microscope photomicrographs of cat CB showing HA-ir staining in glomus cells. (A) HA-ir in electron-opaque granules in the cytoplasm of a glomus cell; bar, 2 μm. (B) Photomicrograph took at high magnification of the electron dense granules of HA-ir; bar, 500 nm. *Inset*: High-magnification section showing mitochondria, dense-core vesicles and granular reticulum in a glomus cell; bar, 500 nm. Note that the protocols used for detection of HA preserve the ultrastructure of the glomus cells.

3.5. Effects of exogenous histamine on carotid sinus nerve discharges

In the perfused cat CB preparation, the application of histamine hydrochloride (0.5–2000 μg in boluses of 200 μl) produced a dose-dependent increase of f_{CSN} (Fig. 4A). The threshold of the responses was near 1 μg and the maximal response was obtained for doses of 500 μg. The excitation lasted for 1–2 min. Similarly, in the superfused cat CB preparation, the application of histamine increases f_{CSN} in a dose-dependent manner (Fig. 4B). However, the threshold of the responses was higher (50 μg) and the excitatory effect was prolonged, probably because histamine has to diffuse to the core of the CB. When testing the responses to repeated applications of histamine hydrochloride to the perfused or superfused CB, transient desensitization was observed, especially for doses above 100 μg. Normally, a 25 min interval was required between consecutive applications of the same dose to achieve similar neural responses. On the contrary to what we observed in the CB preparations, the application of histamine hydrochloride (0.5–2000 μg) to the isolated PG was ineffective in modifying f_{CSN}. Fig. 4C shows that a bolus injection of 250 μg of histamine applied to one isolated PG had no effect on f_{CSN}, while the applications of 250 μg of ACh increased f_{CSN}, as previously reported (Alcayaga et al., 1998; Iturriaga et al., 2007).

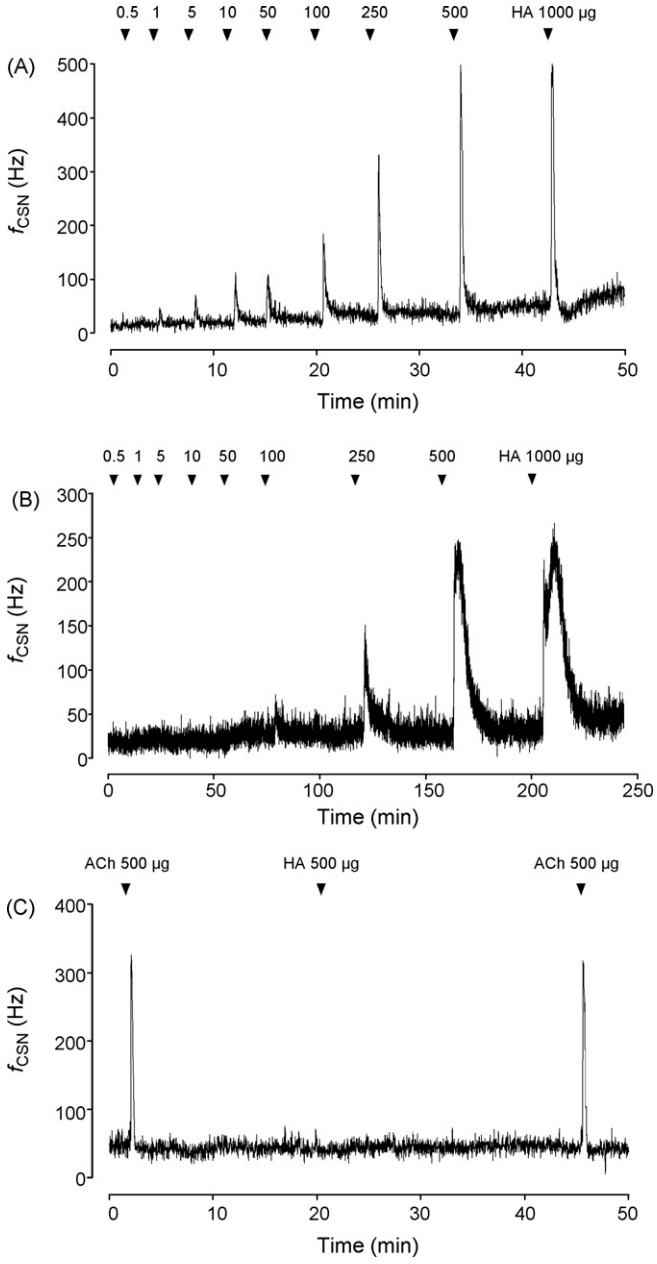

Fig. 4. Effects of histamine on chemosensory activity. Exogenous application of histamine hydrochloride (HA 0.5–1000 μg) to 1 perfused CB (A), 1 superfused CB (B) and 1 isolated petrosal ganglion (C). In A and B, histamine application (arrowheads) increased the frequency of discharge in the carotid sinus nerve (f_{CSN}). Application of histamine to the somata of PG neurons failed to modify CSN discharges, while ACh increases f_{CSN}. f_{CSN}, expressed in Hz.

Fig. 5 compares the excitatory effect of histamine hydrochloride (10 and 50 μg), nicotine (5 μg) and hypoxic perfusion ($P_{O_2} \sim$ 25 Torr) on CSN discharges in a perfused cat CB. Hypoxia as well as 5 μg of nicotine produced maximal excitation, increasing f_{CSN} up to near 500 Hz (Fig. 5A). On the contrary, 10 and 50 μg of histamine hydrochloride increased f_{CSN} up only to 250 Hz (Fig. 5B). Clearly, nicotine was more effective than histamine to increase f_{CSN}. Fig. 5C shows the effects of comparable doses of nicotine and histamine, expressed in nmol on Δf_{CSN}. Undoubtedly, the maximal excitatory response induced by nicotine was obtained at lower doses com-

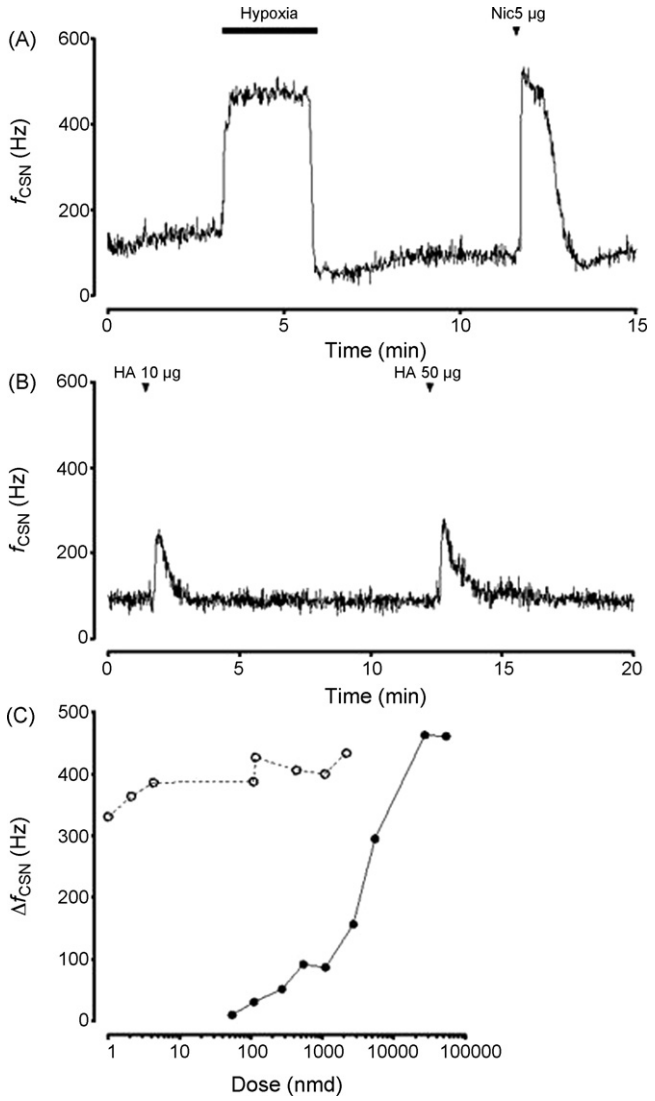

Fig. 5. Comparison of the effects of nicotine, hypoxia and histamine on the chemosensory responses in 1 perfused cat CB. (A) Effects of nicotine (5 μg) and hypoxic perfusion ($P_{O_2} \sim 25$ Torr) on f_{CSN}. (B) Effect of histamine (10 and 50 μg) on f_{CSN}. (C) Dose–response curves for the effects of comparable molar doses of nicotine (○) and histamine (●) on Δf_{CSN}.

pared to histamine. Fig. 6 summarizes the dose–response effects of histamine hydrochloride (0.5–2000 μg) on f_{CSN} applied to perfused and superfused cat CB preparations, and to the isolated PG. In the perfused CB preparation, histamine hydrochloride at doses of 500 μg elicited maximal responses, similar to those attained with hypoxic perfusion or nicotine. The ED_{50} for the excitatory effect of histamine on the perfused CB chemosensory discharges was 128.0 ± 0.6 μg ($n = 4$), while in the superfused CB preparation, the ED_{50} for the excitatory effect was 779.5 ± 1.81 μg ($n = 6$). Note that doses of histamine hydrochloride as large as 2000 μg were ineffective to produce any change in f_{CSN} when were applied to the isolated PG (Fig. 6; $n = 5$).

3.6. Pharmacological effects of histamine antagonists on the chemosensory excitation induced by histamine

In the perfused CB, the application of the antagonist pyrilamine (10–100 μM) in the perfusate medium produced a sustained and progressive increase of CB chemosensory discharges. Indeed, perfusion with Tyrode containing pyrilamine (10–100 μM) increased f_{CSN} from 50 ± 10 to 370 ± 47 Hz ($p < 0.05$; $n = 4$). The increased chemosensory discharge was transiently reduced by injections of Tyrode containing the nitric oxide donor NOC-9 into the carotid artery, suggesting that pyrilamine produced a vasoconstriction in the CB (not shown). Thus, to study the effects of histamine antagonist we used the superfused preparation of the CB, which is devoid of direct vascular effects (Eyzaguirre and Koyano, 1965). Accordingly, we tested the effects of pyrilamine on chemosensory responses elicited by histamine in 5 superfused CBs. Superfusion with Tyrode containing 10–100 μM pyrilamine produced a complete block within 1 h of the excitatory responses evoked by histamine, but also blocked the response elicited by ACh (0.1–500 μg). Fig. 7 shows the inhibitory effect of 100 μM pyrilamine on the chemosensory response elicited by a large dose of histamine (1000 μg) in a superfused CB. Note that following the superfusion with pyrilamine, the chemosensory response elicited by 50 μg ACh was also abolished. The inhibitory effect of pyrilamine was associated with a partial block of conduction in fibers of the CSN. As is shown in Fig. 7B (inset), 100 μM pyrilamine reduced the amplitude of the compound action potential of the CSN slow conducting fibers. Upon removal of pyrilamine, the amplitude of the compound action potential was restored (Fig. 8). Since 100 μM pirylamine produced an anesthetic effect on the conduction of action potentials in the CSN, we lowered its concentration to 500 nM. Fig. 9 shows the effect of superfusion of Tyrode with 500 nM pyrilamine on the chemosensory response elicited by histamine 1000 μg and ACh 100 μg. We found that the excitatory response induced by histamine was reduced, while the response induced by ACh was not affected. Fig. 10A summarized the effects of 500 nM pyrilamine on the chemosensory response evoked by histamine in 4 superfused CBs. The response elicited by 250 μg histamine was significantly reduced ($p < 0.05$) by 500 nM pyrilamine, while the response elicited by 50 μg ACh was not affected. We also tested the effects of the H2 receptor blockers cimetidine and ranitidine on the excitatory chemosensory response elicited by histamine in the superfused CB preparation. Superfusion with Tyrode containing the H2 blocker cimetidine (50 μM, Fig. 10B) for 1 h did not affect the chemosensory responses elicited by 250 μg histamine. We also

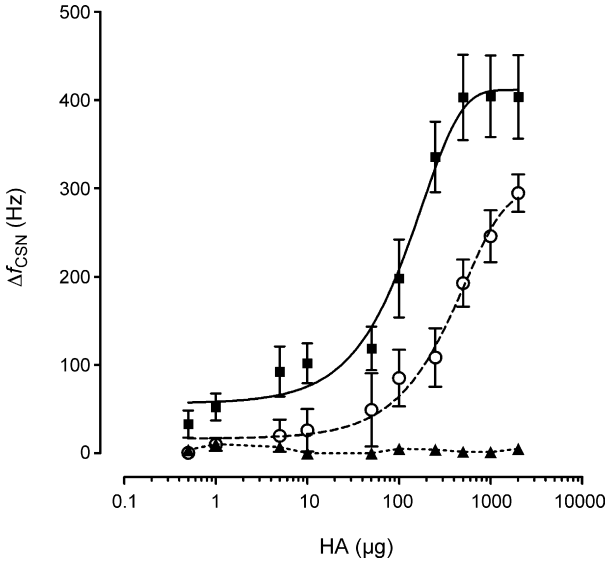

Fig. 6. Dose–response curves for the effects of histamine (HA) on chemosensory discharges in 4 perfused CBs (■), 6 superfused CBs (○) and 5 isolated PGs (▲). Δf_{CSN}, changes in carotid sinus nerve frequency discharge. Dose–response curves are statistically different ($p < 0.05$, two-way ANOVA).

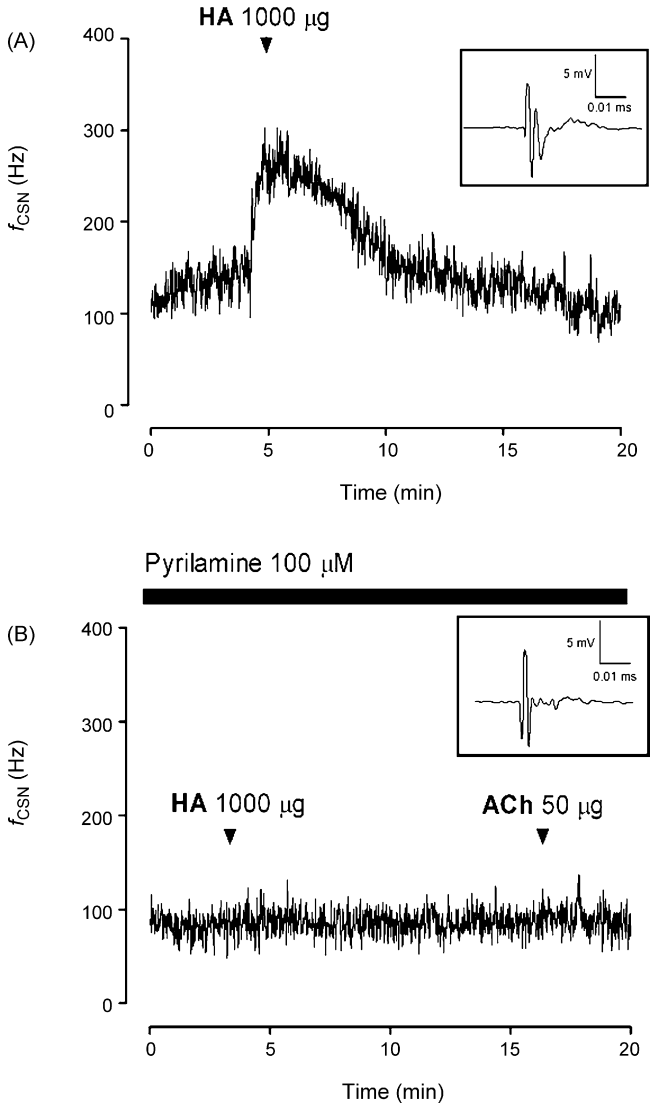

Fig. 7. Effects of 100 μM pyrilamine on f_{CSN} in 1 superfused CB. (A) Histamine increased f_{CSN}. (B) Superfusion of Tyrode containing 100 μM pyrilamine abolished the response evoke by 1000 μg histamine and by 50 μg ACh. *Insets*: In A and B show the evoked compound action potential elicited by electrical stimulation of the CB.

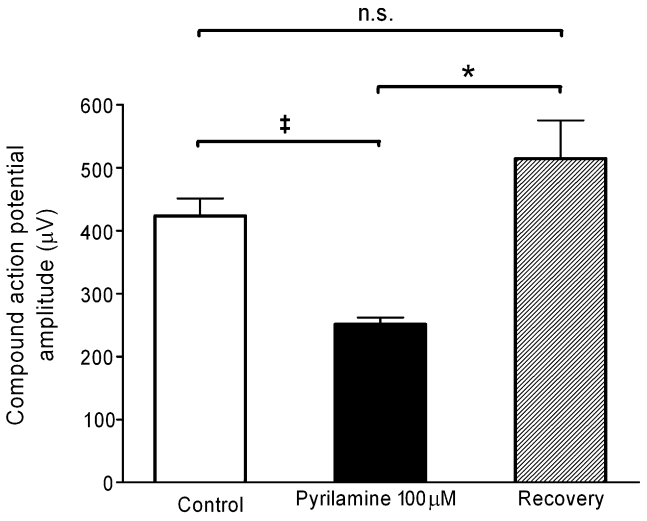

Fig. 8. Quantification of effects of 100 μM pyrilamine on the CSN compound action potential in 3 superfused CBs. ($^{‡}p < 0.01$, $^{*}p < 0.001$, Bonferroni test after one-way ANOVA, $n = 82$ action potentials; n.s., no statistical differences).

found that the superfusion of the H2 blocker ranitidine (100 μM) did not modified the response elicited by 250 μg histamine. In 3 superfused cat CB preparations, 250 μg histamine produced similar increases of chemosensory discharges in the absence and presence of 100 μM ranitidine (0.90 ± 0.13 vs 0.78 ± 0.12 $\Delta f_{CSN}/\Delta f_{max}$, control and during ranitidine, respectively, $p > 0.05$, paired *T*-test). On the contrary, superfusion with Tyrode containing 30 μM thioperamide, a H3 antagonist, enhanced the chemosensory responses elicited by 250 μg histamine (Fig. 11). In 4 superfused cat CB preparations, 250 μg histamine produced a significantly ($p > 0.05$; paired *T*-test) larger increase of chemosensory discharges in the presence of 30 μM thioperamide ($67.1 \pm 7.8\%$) than in control conditions ($50.5 \pm 5.1\%$), with respect to the maximal frequency discharge induced in each preparation by ACh.

4. Discussion

Present results provide new information regarding the subcellular localization of histamine in glomus cells of the cat CB and its effects on CSN discharges. Double immuno-staining for histamine and TH shows that CB glomus cells contain HA-ir. Moreover, the electron microscopic study showed that HA-ir in the cytoplasm of glomus cells is associated with dense-cored vesicles. In addition, we found that application of exogenous histamine produced a dose-dependent chemosensory excitation in the CB preparations, but has no effect on the isolated PG. In the superfused CB preparation, 500 nM pyrilamine, reduced the chemosensory excitation induced by histamine, but the H2 antagonist cimetidine (50 μM) or ranitidine (100 μM) did not modify the excitatory response induced by histamine. Thus, present results suggest that cat glomus cells contain histamine and that its exogenous application produces a dose-dependent chemosensory excitation in the CB, while being ineffective when applied to the soma of PG neurons.

Landgren et al. (1954) found that histamine has no effect on CSN discharges when was applied to the cat CB *in situ*. They reported that doses of 2–50 μg histamine hydrochloride did not increase chemosensory discharges during oxygen breathing, while 2 μg ACh produced a considerable chemosensory excitation. Nevertheless, Landgren et al. (1954) recorded the neural activity from the entire CSN, consisting of both chemosensory and barosensory discharges; the latter may obscure the effects of histamine on chemosensory discharges. On the other hand, if histamine has the same effect on the CB territory that on the cat hindlimb (Champion and Kadowitz, 1997), the profound vasodilation may modify the chemosensory response. Therefore, to study the effects of histamine on CB chemosensory discharges, it is essential to use CB preparations without the interference of systemic effects. Thus, to assess the effects of histamine we used perfused and superfused preparations *in vitro* of the cat CB. In both preparations, histamine hydrochloride produced a potent dose-dependent excitatory effect on chemosensory discharges. In the perfused CB preparation, with an intact vascular network, histamine hydrochloride produced a similar maximal chemosensory excitation as compared with those evoked by nicotine or hypoxia. However, the cat CB was more sensible to nicotine than histamine. Indeed, maximal effect on chemosensory discharges was obtained with lower doses of nicotine than histamine (Fig. 4). This observation may explain why Landgren et al. (1954) found that 2–50 μg histamine hydrochloride failed to evoke an appreciable chemosensory response in the whole animal. In the superfused, CB preparation, large doses of histamine are needed to evoke chemosensory excitation, but in this preparation, histamine has to diffuse from the surface to the core of the CB.

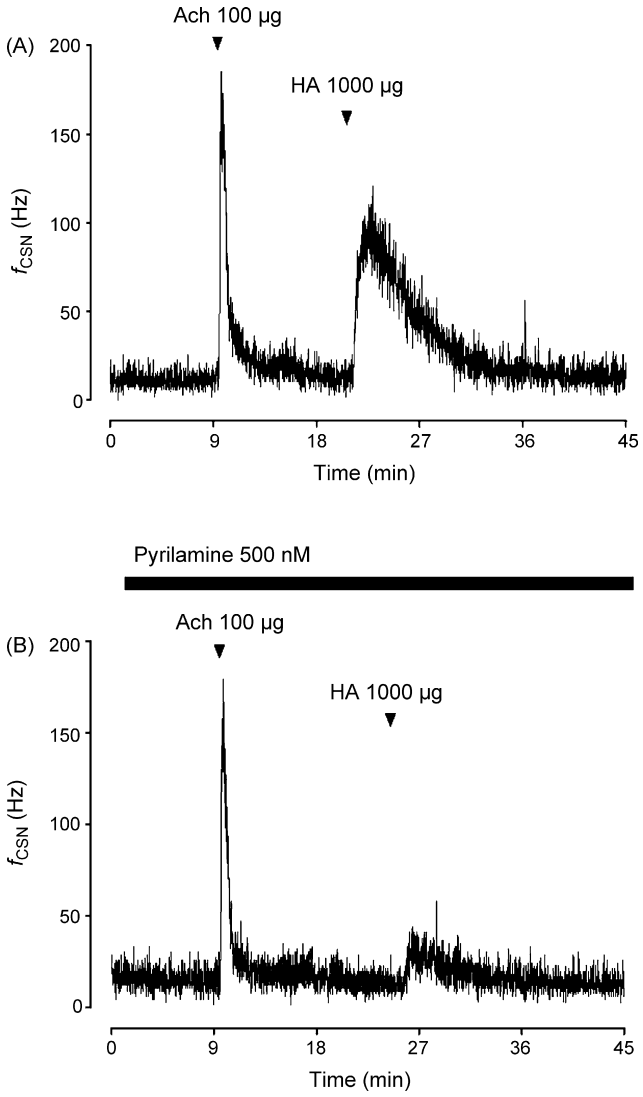

Fig. 9. Effects of 500 nM pyrilamine on f_{CSN} in 1 superfused CB. (A) Histamine increased f_{CSN}. (B) Superfusion of Tyrode containing 500 nM pyrilamine reduced the response evoke by 1000 µg histamine, without affecting the response evoked by 100 µg ACh.

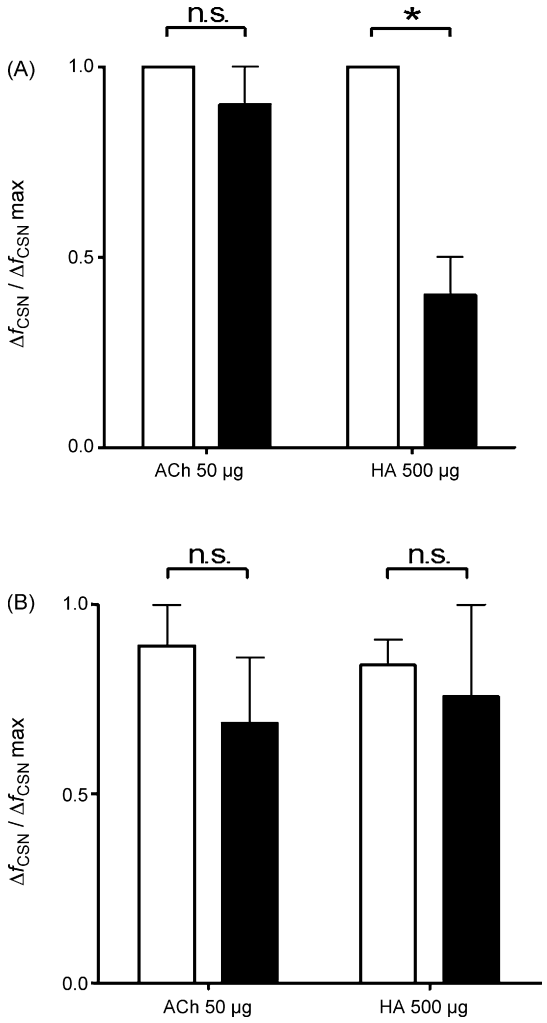

Fig. 10. Effects of pyrilamine and cimetidine on chemosensory responses evoked by histamine and acetylcholine in the superfused CB. (A) Effect of 500 nM pyrilamine on the chemosensory responses evoked by 500 µg histamine and 50 µg acetylcholine on 4 cat CBs superfused *in vitro*. 500 nM pyrilamine superfused continuously to the CB did not modify the responses evoke by 500 µg ACh, but significantly reduced the response induced by histamine ($p < 0.01$, Bonferroni test after two-way ANOVA). (B) Effect of 50 µM cimetidine on the response induced by 250 µg histamine in 2 superfused CB. Cimetidine (50 µM) did not modify the response evoke by histamine (n.s., no statistically significant differences). Empty bars, control. Filled bars, HA antagonists.

The excitatory response induced by histamine in the cat CB appears not to be due to a reduction of the P_{O_2} in the CB parenchyma, and a consequent increase of chemosensory discharges. The blockade of all histaminergic receptors with a large dose of pyrilamine (100 µM) in the perfused CB increase chemoreceptor discharges to approximately maximal level, effect transiently abolished by a NO donor, supporting the idea that histamine may act as a local tonic vasodilatator in the CB, as it does in other vascular territories in the cat (Champion and Kadowitz, 1997). On the other hand, using a superfused CB preparation, we found that histamine increased the chemosensory discharges, indicating that histamine excitatory effects on the CB are not mediated by its vascular actions.

Koerner et al. (2004) proposed histamine as an excitatory transmitter between glomus cells of the CB and nerve terminals of the PG neurons. They found that the rat CB releases histamine in response to hypoxia, and expresses the components for its biosynthesis and storage, as well as the H1, H2 and H3 histamine receptors mRNAs. Thus, histamine fulfills some criteria to be considered a transmitter in the CB (Eyzaguirre and Zapata, 1984). In addition to the findings of Koerner et al. (2004), we found that histamine is associated to dense-cored vesicles in the cytoplasm of glomus cells, and the application of exogenous histamine mimics the effects induced by natural stimuli. Since histamine did not excite the cat PG neurons as other putative transmitters do (Alcayaga et al., 1998, 2000; Iturriaga et al., 2007; Zhang et al., 2000; Zhong and Nurse, 1997), it appears that its primary excitatory effects are mediated by histamine receptors located on the cat CB parenchymal cells, suggesting that histamine plays an autocrine and/or paracrine role in the generation of the chemosensory activity in the cat CB. Another possibility is that histamine may act as a modulator of the excitatory responses in the PG terminals. For instance, the CB putative transmitter dopamine has no direct action on f_{CSN} when applied to the isolated PG, but modulates the responses induced by ACh and ATP (Alcayaga et al., 1999b, 2003) and the PG responses to cytotoxic hypoxia (Alcayaga et al., 1999a) when applied 30 s in advance. However, although present data shows no indication of responses to histamine applied to the soma of the cat PG neurons, we cannot rule out the possibility that physiological effects at the PG postsy-

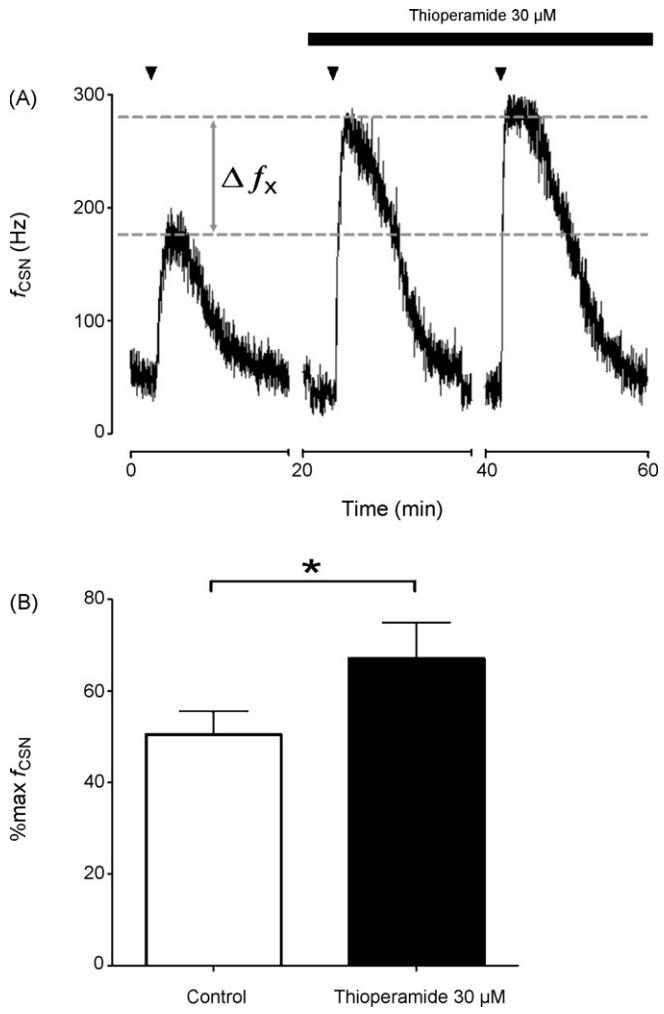

Fig. 11. Effect of thioperamide on chemosensory responses evoked by histamine in the superfused CB. (A) Effect of 30 μM thioperamide on the excitatory chemosensory response induced by 250 μg histamine (▼) in 1 superfused cat CB. Histamine-induced increase of chemosensory discharge was augmented by about 50% (segmented line) during thioperamide superfusion (continuous bar). (B) Effect of 30 μM thioperamide on the chemosensory responses evoked by 250 μg histamine on 4 CBs ($p < 0.05$, paired T-test).

naptic level may occur in other species. In fact, Lazarov et al. (2006) found immunoreactivity for H1 and H3 histamine receptors, as well as histidine decarboxylase in the rat PG neurons. Thus, differences on the action of exogenously applied histamine and its participation on the generation of afferent chemosensory activity may reflect true species differences (see Iturriaga et al., 2007 review). Other afferent primary neurons also present histamine receptors. In the guinea pig, about 15% of the trigeminal and lumbar dorsal root ganglion neurons are endowed with H1 receptors, while none of the nodose ganglion neurons expresses H1 receptors (Kashiba et al., 1999). Present data shows no indication of sensitivity to histamine applied to the soma of PG neurons, but cannot rule out the possibility that histamine may act at the level of the nerve endings in the CB. On the other hand, we cannot rule out the possibility that histamine may excite the nerve terminals of the PG neurons, but not their somata. However, the soma of PG neurons has been used as a model to study the electrophysiological properties of the afferent ending (Donnelly, 1999; Varas et al., 2003), with the underlying assumption that the soma and the nerve endings share similar membrane characteristics. Several electrophysiological properties are shared by the membranes of peripheral nerve endings and the soma of primary afferent neurons (Gold et al., 1996; Reichling et al., 1997). In addition, immunocytochemical studies have shown that PG neurons express the same nicotinic and purinergic receptors in both the soma and nerve endings (Iturriaga et al., 2007). Since histamine did not excite the cat PG neurons, it is plausible that its excitatory effect in the CB could be the result of an autocrine or paracrine mechanism mediated by H1 receptors located in the glomus cells. Histamine H1 receptor belongs to the G protein-coupled receptor family, leading to the activation of several signaling pathways that increase the intracellular [Ca^{2+}] (Hill et al., 1999). Thus, histamine may increase the intracellular [Ca^{2+}] in glomus cells, as has been found in many other cells (Hill et al., 1999). Moreover, histamine induces the release of catecholamine from chromaffin cells (Livett and Marley, 1986) and stimulates the phosphorylation of TH, mediated by an increase of intracellular [Ca^{2+}] (Bunn et al., 1995). The secretion of histamine from glomus cells may also regulate the excitability of other glomus cells, modifying the oxygen sensing threshold and the secretion of the transmitters. Accordingly, tonic release of histamine under normoxic conditions may modulate the membrane potential of glomus cells acting on background K^+ channels (Buckler, 2007), which are present in glomus cells plasmatic membrane, through activation of H1 receptors. A similar mechanism has been found in neurons. Indeed, the activation of H1 receptor in neurons reduces a background leakage K^+ current (Reiner and Kamondi, 1994; Jafri et al., 1997). In response to hypoxic stimulation, increase histamine release from glomic cells could modulate the CB vascular tone, neighboring glomus cells excitability and release of excitatory transmitters.

We found that 10–100 μM pyrilamine blocked the chemoexcitatory response evoked by histamine in the superfused CB, but also abolished the response elicited by ACh. Certainly, we cannot preclude that a high concentration of pyrilamine (10–100 μM may act as an anticholinergic. In fact, Orzechowski et al. (2005) reported that the pA2 value for pyrilamine as an anticholinergic is 4.8 (KB of 15.8 μM) in two functional bioassays. However, our data clearly showed that perfusion with Tyrode containing 10–100 μM pyrilamine, which produced a complete block of the excitatory responses induced by histamine and ACh (0.1–500 μg), was associated with reduction of the evoked compound action potential of the carotid sinus nerve. Indeed, at these large doses, pyrilamine reduced the amplitude of the compound action potential of the CSN slow conducting fibers, indicating an anesthetizing effect. Similarly, Landgren et al. (1954) reported that intravenous injection of large doses of the histaminergic antagonist lergitin (10 mg) blocked the chemosensory response to hypoxia, effect that they attributed to a local anesthetic action of the drug. Using a lower concentration of pyrilamine (500 nM), we found that the chemosensory response induced by histamine was reduced, while the response elicited by ACh was not significantly affected. We found no evidence of modification of the excitatory chemosensory response elicited by histamine in the superfused CB preparation by the H2 receptor blockers cimetidine and ranitidine. However, thioperamide, a H3 receptor antagonist, enhanced the chemosensory responses elicited by histamine. Thus, our data suggests that histamine exerts its excitatory effects in the CB primarily via H1 receptors. The H3 receptor blockade enhances the response to histamine, probably due to its reported presynaptic inhibitory action in histaminergic terminals (Hill et al., 1999). Thus, histamine may work as an excitatory modulator on the chemosensory process at the level of the glomus cells acting on H1 receptors. This interpretation is supported by the fact that Lazarov et al. (2006) and Koerner et al. (2004) found that H1 and H3 are the predominant histamine receptors expressed in the glomus cells. Lazarov et al. (2006) also found the expression of H3 receptors on dendritic process and perikarya of PG neuron in rats. Thus, it is likely that histamine may

differentially modulate the chemosensory process via H3 autoreceptors at the presynaptic level in the glomus cells, as well as at the postsynaptic level in PG neurons. However, if H3 receptors are present in cat PG terminals in the CB, our data indicates that their activation does not lead to increases in chemosensory afferent activity.

In summary, our data showing that histamine is present in dense-core vesicles of the glomus cells of the CB are compatible with the idea that histamine is release from the glomus cell with other modulators and transmitters in response to hypoxia, and play an autocrine and/or paracrine role in the generation of the chemosensory activity in the cat CB.

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