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Petrosal ganglion responses to acetylcholine and ATP are enhanced by chronic normobaric hypoxia in the rabbit

Gabriel Icekson, Claudia V. Dominguez, Valentina P. Dedios, Jorge Arroyo, Julio Alcayaga*

Laboratorio de Fisiología Celular, Facultad de Ciencias, Universidad de Chile, Santiago, Chile

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ABSTRACT

In mammals, adaptation to chronic hypoxia requires the integrity of the arterial chemoreceptors, specially the carotid body (CB). Chronic hypoxia increases the sensibility of the CB by acting on the receptor cells, but there is limited information on the effects of chronic hypoxia on the sensory neurons that innervate the CB. Therefore, we studied the responses evoked by ACh and ATP, the main transmitters that generate the chemoafferent activity, on the petrosal ganglion (PG) of rabbits exposed to chronic normobaric hypoxia (CNH) during fourteen days. ATP and ACh increased the activity of PG neurons in a dose-dependent manner, in a similar way than in rabbits not exposed to hypoxia (naïve). However, the duration of the responses were significantly increased by CNH, with the mean maximal responses to ACh and ATP increased by a factor of two and four, respectively. Our results suggest that CNH increases duration of the responses by modifying the expression and/or content of ACh and ATP receptors.

1. Introduction

In mammals, the tissue oxygen supply is controlled by reflex cardiovascular and ventilatory mechanisms that match oxygen supply with oxygen consumption. Acute hypoxia produces a brisk increase in tidal volume and/or ventilation frequency, response termed hypoxic ventilatory response (HVR), which is peripherally driven by the arterial chemoreceptors (Gonzalez et al., 1994; Teppema and Dahan, 2010). On the other hand, long lasting hypoxia increases basal ventilation progressively until the ventilation stabilizes at a higher level (Powell et al., 1998), phenomenon known as hypoxic ventilatory acclimatization (HVA). Moreover, during the HVA the HVR increases, indicating an increase in sensibility and/or reactivity of the ventilatory control. Initially the HVA was postulated to be originated within the central nervous system (Severinghaus et al., 1963) without the participation of the peripheral chemoreceptors (Sørensen, 1970), but it has been shown that peripheral chemoreceptors are necessary for the development of HVA (Smith et al., 1986). Denervation of the carotid bodies reduces basal ventilation and precludes the development of the HVA (Forster et al., 1981; Smith et al., 1986). In animals exposed to chronic hypobaric hypoxia the HVR is significantly larger than in control animals (Aaron and Powell, 1993), and the carotid sinus nerve single fiber basal activity (Vizek et al., 1987) as well as the responses to acute hypoxia are increased (Barnard et al., 1987; Vizek et al., 1987), without changes in the responses induced by anoxia or hypercapnia, suggesting an increased gain of the chemoreceptor to hypoxia.

Recordings from rat carotid bodies *in vitro*, exposed to hypobaric hypoxia for 3–20 days, show an increased frequency discharge under basal conditions and an increased response to acute hypoxia (Chen et al., 2002a; He et al., 2005, 2006). Carotid body cells of rats gestated and reared hypoxic for 5–10 days, present K^+ currents that are depressed by hypoxia as in control animals (Hempleman, 1995, 1996), although the current density could be reduced because of hypertrophy of glomus cells (McGregor et al., 1984; Stea et al., 1992). In glomus cells from adult rabbits cultured in hypoxic media for 24–48 h, acute hypoxia inhibits the O_2-sensitive K^+ current to a larger extent than in cells obtained from control animals, producing a larger depolarization (Kääb et al., 2005). Similarly, TTX-sensitive sodium (Caceres et al., 2007; Stea et al., 1992, 1995) and nifedipine-sensitive (L-type) calcium currents (Hempleman, 1995, 1996) are increased in magnitude and density by chronic hypoxia with respect to control cells, although no modification of the calcium current density has also been reported (Peers et al., 1996; Stea et al., 1992, 1995). These data suggests that chronic hypoxia increases glomus cells excitability and exocytotic release of transmitters, increasing in that way the afferent discharge.

It is generally accepted that depolarization of glomus cells induce the release of one or several transmitters that, acting both in pre- and postsynaptic receptors generate and/or maintain the afferent chemosensory activity (Gonzalez et al., 1994; Iturriaga and Alcayaga, 2004; Nurse and Piskuric, 2013). Acetylcholine (ACh)

* Corresponding author at: Laboratorio de Fisiología Celular, Facultad de Ciencias, Universidad de Chile, Casilla 653, Santiago, Chile. Tel.: +56 2 2978 7366; fax: +56 2 2271 2983.
E-mail address: jalcayag@uchile.cl (J. Alcayaga).

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and ATP play a major role in the generation of the afferent discharge (Conde et al., 2012; Iturriaga and Alcayaga, 2004; Nurse and Piskuric, 2013). Afferent discharge of identified cat petrosal chemosensory neurons increases by stop-flow or acidification of the carotid body, responses partially blocked by either a nicotinic receptor antagonist (mecamylamine) or a nucleotide receptor antagonist (suramin), and completely blocked by the join application of the antagonists (Varas et al., 2003). Similar responses have been recorded in co-cultures of rat petrosal ganglion neurons and carotid body tissue (Nurse and Zhang, 1999; Prasad et al., 2001; Zhang et al., 2000). These responses are mediated by receptors located on the petrosal ganglion neurons (Alcayaga et al., 1998, 2000, 2007; Prasad et al., 2001; Shirahata et al., 1998; Soto et al., 2010; Zhang et al., 2000).

Both the basal discharge and the responses to acute hypoxia are increased in the peripheral chemoreceptors after HVA, indicating an increased sensibility, but the mechanisms underlying this increased sensibility are still not fully understood. Because a change in synaptic efficacy in the carotid body may be partly responsible for the increased gain, we hypothesized that chronic hypoxia could increase the responses of petrosal ganglion neurons to the carotid body putative transmitters, thus increasing the responses of the afferent arm of the reflex pathway. To test our hypothesis, we studied the responses evoked by ACh and ATP in petrosal ganglia obtained from male adult rabbits exposed to normobaric hypoxia ($PO_2 \sim 75$ mm Hg) for approximately 15 days.

2. Methods

2.1. Animals

Experiments were performed in 7 male New Zealand white rabbits. Experiments were conducted in accordance to the guidelines of the Fondo Nacional de Desarrollo Científico y Tecnológico (FONDECYT, Chile) and the Bio-Ethics Committee from the Facultad de Ciencias of the Universidad de Chile approved the experimental protocols.

2.2. Chronic normobaric hypoxia exposure

On each experiment, two individually caged (D × H × W; 0.55 × 0.42 × 0.38 m) animals were exposed to normobaric hypoxia for 14.4 ± 0.4 days ($n = 7$) in a 300 L (0.6 × 0.5 × 1.0 m) sealed plastic chamber. The O_2 content was continuously measured with an oxygen sensor (AX300, Teledyne Analytical Instruments, USA) which output was connected to an automatic programmable controller (Zelio SR2 B121BD, Schneider Electric, France), which maintained the oxygen content (F_{IO_2}) around 9.1%. The controller opened solenoid valves (2026BV172, Jefferson Solenoid Valves, USA), admitting compressed air or N_2 to the chamber if F_{IO_2} values were below ~8.2% or over ~10.4%, respectively, and simultaneously a relief valve that remained opened for 40 s after the closure of the admitting ones. Additionally, two mechanic relief valves opened whenever the gauge pressure exceeded 17 mm Hg inside the chamber. Four internal fans homogenized the atmosphere in the chamber. Ventilatory CO_2 was trapped using $CaCO_3$ (250 g) and the urinary ammonia with H_3BO_3 (60 g). Every other day, the chamber was opened for about 5 min to clean the cages and chamber walls and replenish the food and water containers; $CaCO_3$ and H_3BO_3 were changed when necessary. The chamber internal pressure was measured continuously with a gauge transducer and along with the F_{IO_2} signal was recorded (WinDaq, DATAQ Instruments, Inc., USA) at 1 Hz with an analog to digital acquisition system (DI-158U, DATAQ Instruments, Inc., USA). Throughout the hypoxic period the temperature and the relative humidity within the chamber were recorded at 5 min intervals with a data logger (EL-USB-2, Lascar Electronics Inc., USA).

2.3. Physiological recordings

Male New Zealand white rabbits were anesthetized with a ketamine/xylazine mixture (75/7.5 mg/kg, i.m.), with additional intramuscular doses (1/3 of the initial dose) applied when necessary to maintain the surgical anesthetic level. The animals were placed in supine position and the glossopharyngeal nerve dissected through an incision in the midline of the neck, exposing the carotid bifurcation and the carotid nerve was severed close to the CB. The PG was exposed by eroding the tympanic bulla and the petrosal bone and the glossopharyngeal nerve was cut cephalically to the central apparent limit of the ganglion. The ganglion with its attached nerves was placed in ice-chilled Hanks' balanced salt solution (HBSS), and the surrounding connective tissue removed from over the ganglion and along the full extension of the nerves. The ganglion was placed over a pair of stimulating electrodes, secured to the bottom of a 0.2 mL chamber, and superfused with air-equilibrated HBSS supplemented with 5 mM HEPES buffer, pH 7.4 at 38 ± 0.5 °C, flowing at 1.2–1.5 mL/min.

The carotid nerve was placed on paired platinum–iridium electrodes, and covered with mineral oil in an upper compartment of the superfusion chamber. The electrodes were connected in turn to an AC-preamplifier (Model 1800, A-M Systems, USA) and the recorded electroneurogram was amplified, displayed in oscilloscope, and stored in videocassette tape after digital encoding. The electroneurogram was also fed to a spike amplitude discriminator which standardized output pulses were digitally counted to assess the carotid nerve frequency of discharge (f_{CN}), in Hz. The temperature of the chamber was monitored with a thermistor (YSI, USA) and with the f_{CN} were digitized (DI-145, Dataq Instruments Inc., USA), displayed and recorded (WinDaq, Dataq Instruments Inc., USA) at 1 Hz. Bethanechol, ACh and ATP, in doses of 1–1000 μg in 10 μL boluses, were applied over the ganglion.

2.4. Data evaluation

The change in frequency of discharge (Δf_{CN}) was calculated as the difference between the maximal frequency achieved during a single response and the mean basal frequency (bas f_{CN}), computed in a 30 s interval prior to every evoked response. The relation between Δf_{CN} and the doses of any of the used drugs was assessed by fitting the evoked responses to a sigmoid curve ($\Delta f = \Delta f_{max}/[1 + (ED_{50}/D)^S]$), where D, applied dose; ED_{50}, the dose that evoked half-maximal response, and S, Hill slope factor determining the steepness of each curve. Duration of the responses (Δf_D) was assessed measuring the time f_{CN} remained increased over the 99% confidence limit of the bas f_{CN}, and were related to the dose with a sigmoid curve ($\Delta f_D = \max \Delta f_D/[1 + (ED_{50}/D)^S]$), where max Δf_D, corresponds to the maximal duration of the response. Curve fitting was performed using CurveExpert Professional© (version 1.6.3, Daniel G. Hyams). Comparisons between responses of animals exposed CNH and animals not exposed to hypoxia (naïve) were assessed using our previously published data (Soto et al., 2010).

2.5. Atmospheric variables

Yearly barometric pressure values for Santiago (717.2 ± 0.3 mm Hg; altitude, 567 m) and relative humidity (63.3 ± 2.5%) were obtained from the web database (http://164.77.222.61/climatologia/) of the Dirección Meteorológica de Chile of the Dirección General de Aeronáutica Civil.

2.6. Statistical analysis

All data is presented as mean ± SEM. Statistical analysis of data was performed with GraphPad Prism (version 5.04 for Windows, GraphPad Software, San Diego California, USA). Differences between samples were assessed using Student's *t*-test or two-way ANOVA, depending on the data structure. Significance of correlation between variables was assessed using Student's *t*-test. The significance level was set at $P < 0.05$ for all statistical analyses.

3. Results

3.1. Environmental variables within the chamber during CNH exposure

The initial atmospheric F_{IO_2} (20.98 ± 0.13%; $n = 5$) was reduced and regulated at a mean F_{IO_2} value of 9.07 ± 0.03% (range, 8.20 ± 0.14%–10.42 ± 0.11%; $n = 5$ periods of 24 h). The admission of air (or N_2) into the chamber, with a mean inter-admission interval of 13.7 ± 2.6 min ($n = 15$ consecutive admissions), increased the pressure to a mean maximal value of 15.8 ± 0.1 mm Hg ($n = 5$ periods of 24 h). The maximal pressure returned exponentially to basal levels, with time constants of 11.6 ± 0.1 s and 76.0 ± 2.2 s ($r^2 = 0.99$; $P < 0.01$; $n = 15$ consecutive admissions), producing a mean increase of 1.3 ± 0.3 mm Hg ($n = 5$ periods of 24 h) within the chamber. Thus, the mean barometric pressure inside the chamber increased slightly but significantly ($P < 0.05$; Student's *t*-test) to 718.5 ± 0.3 mm Hg.

The mean temperature in the chamber was 22.9 ± 0.4 °C (range, 20.0 ± 0.3 °C–26.0 ± 0.7 °C; 5 periods of 14 days), with no significant differences between consecutive 48 h periods ($P > 0.05$; one way repeated measures ANOVA). Conversely, the minimal relative humidity increased significantly ($P < 0.01$; one way repeated measures ANOVA; $n = 5$) from 50.8 ± 0.9% to 69.8 ± 0.8% at the first opening of the chamber, with no further modification upon consecutive openings ($P > 0.05$; one way repeated measures ANOVA). During the first day of each 48 h cycle the relative humidity attained a mean maximal value of 92.8 ± 0.03% (30 periods of 24 h), further increasing to a significantly ($P < 0.05$; two way repeated measures ANOVA) higher maximal mean value of 97.1 ± 0.5% (30 periods of 24 h) on the second day. Thus, the mean relative humidity inside the chamber was 94.4 ± 0.40% (5 periods of 14 days), significantly higher ($P < 0.01$; Student's *t*-test) than the mean atmospheric relative humidity (63.3 ± 2.5%).

3.2. CNH effects on growth and hematocrit

The initial mean weight of animals (2.07 ± 0.18 kg; $n = 7$) was not significantly modified (1.94 ± 0.16 kg; $P > 0.05$; Student's paired *t*-test) during CNH exposure. Conversely, the hematocrit increased significantly ($P < 0.01$; Student's paired *t*-test; $n = 7$) from 40.5 ± 0.7% to 54.6 ± 1.7% during the CNH period.

3.3. CNH effects on ACh induced responses

Acetylcholine effects were tested in 10 ganglia obtained from 7 animals exposed to CNH. The application of ACH produced a rapid increase of f_{CN} which amplitude and duration was dose dependent. Fig. 1 shows the responses induced by ACh, applied every 5 min, in a single ganglion (Fig. 1A). In this ganglion the responses presented a threshold around 0.5 μg, a half maximal response for doses between 2 and 5 μg and a plateau of maximal activity for doses above 20 μg (Fig. 1A and B). Similarly, the duration of the responses presented its half maximal duration and a plateau of maximal duration in the same range than Δf_{CN} (Fig. 1C). The mean increases in activity (Δf_{CN}) for the ten tested ganglia are depicted in Fig. 2A.

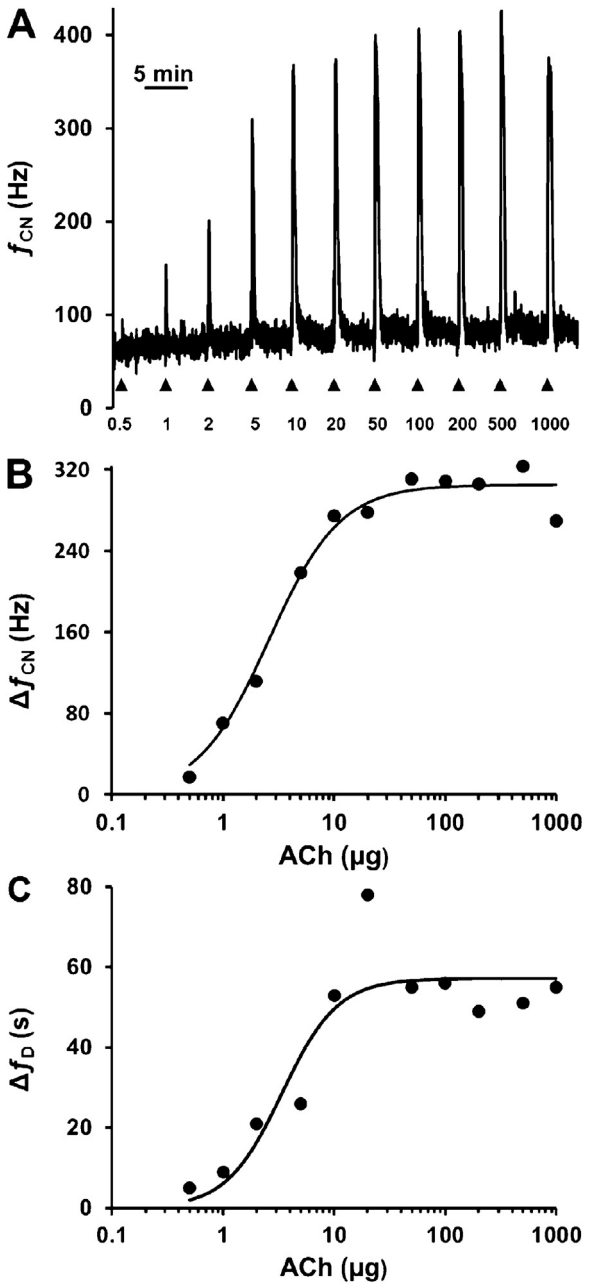
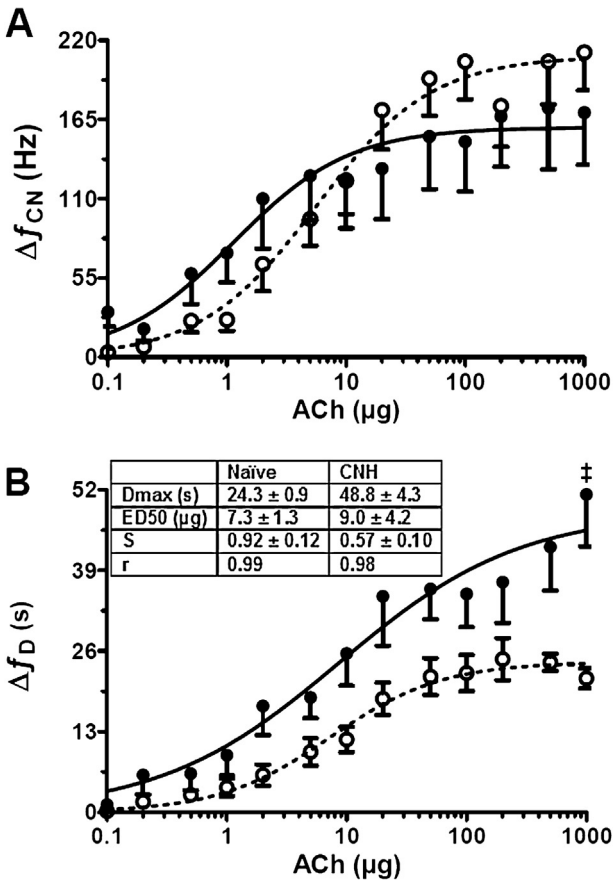


Fig. 1. Responses induced by ACh in a single petrosal ganglion preparation of a rabbit exposed to CNH. (A) Increases in carotid nerve frequency discharge (f_{CN}) evoked by increasing doses of ACh (arrowhead). (B) Dose-response relationship for the increases in the frequency of discharge (Δf_{CN}) in the same preparation. (C) Relationship between the dose and the duration of the responses (Δf_D) induced by ACh in the same preparation.

There were no statistical significant differences ($P > 0.05$, two-way ANOVA) in the Δf_{CN} evoked in ganglia from rabbits exposed to CNH and naïve rabbits. The mean duration of ACh-induced responses ($n = 10$) were significantly correlated ($r = 0.98$, $P < 0.05$ Student's *t*-test) with the applied dose, with an ED_{50} of 9.03 ± 4.19 μg, a slope factor of 0.57 ± 0.10, and a maximal duration of 48.79 ± 4.27 s. The mean duration curve (Fig. 2B) for the rabbits exposed to CNH was significantly different ($P < 0.05$, two-way ANOVA) than the one of naïve rabbits, with significant differences ($P < 0.05$, Fisher *F*-test) in the maximal duration but not in the ED_{50} or the slope factor ($P < 0.05$, Fisher *F*-test).

The responses to consecutive applications of the same ACh dose were of similar amplitude and duration when they were



	Naïve	CNH
Dmax (s)	24.3 ± 0.9	48.8 ± 4.3
ED50 (µg)	7.3 ± 1.3	9.0 ± 4.2
S	0.92 ± 0.12	0.57 ± 0.10
r	0.99	0.98

Fig. 2. Mean effect of ACh on carotid nerve activity in naïve and CNH rabbits. (A) Dose-responses relationship for the mean increases of frequency discharge (Δf_{CN}). No significant differences between the responses of naïve ($n = 13$) and CNH ($n = 10$) rabbits ($P > 0.05$; two-way ANOVA). (B) Relationship between the dose and the duration (Δf_D) of the ACh-induced responses. The curves were significant different between naïve ($n = 7$) and CNH ($n = 10$) rabbits ($P < 0.01$; two-way ANOVA; ‡$P < 0.001$ Bonferroni test). Inset, table with parameters for the fitted curves; Naïve rabbits, empty circles, dotted line; CNH rabbits, filled circles, continuous line. Data from naïve animals obtained from Soto et al., 2010.

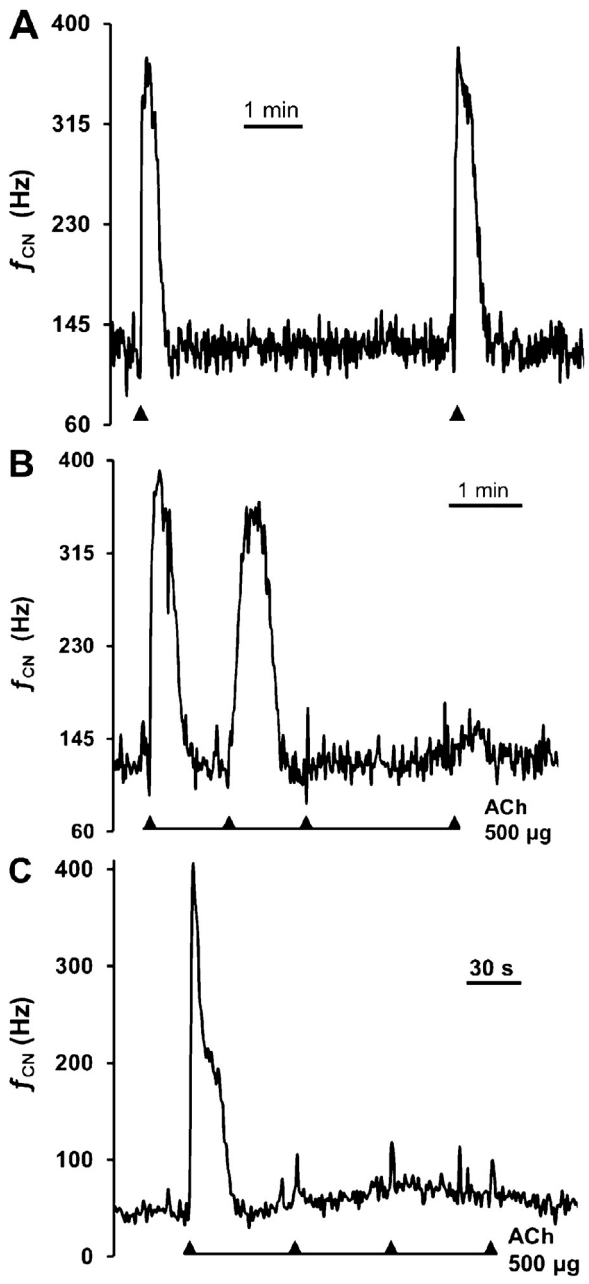


Fig. 3. Temporal desensibilization of ACh-induced responses in petrosal ganglia of CNH rabbits. (A) Two successive applications of a single ACh dose (1000 µg; arrowhead), separated by 5 min, produced responses of similar amplitude and duration. (B) Successive applications of a single ACh dose (500 µg; arrowhead), delivered at 1 min intervals, produced a complete desensitization at the third application that lasted more than 2 min. (C) Successive applications of a single ACh dose (500 µg; arrowhead), delivered at 1 min intervals, produced a complete desensitization at the second application.

delivered every 5 or more min (Fig. 3A). However, responses to the same dose applied in shorter intervals were desensitized to a variable extent (Fig. 3B and C). Moreover, when the same dose was applied repetitively at 1 min intervals, the responses after the second application were almost completely obliterated (Fig. 3B and C). Finally, the application of bethanechol (1–1000 µg) to the petrosal ganglia ($n = 3$; data not shown) had no effect on f_{CN}, and did not modify the responses evoked by successive ACh applications.

3.4. CNH effects on ATP induced responses

The effects of ATP were tested in 8 ganglia obtained from 6 animals exposed to CNH. Application of ATP produced a fast and transient increase in f_{CN}, which amplitude and duration were dose dependent. The responses induced by ATP in a single preparation are depicted in Fig. 4. In this case the responses were evoked by doses over 0.1 µg, reached a plateau of maximal activity for doses over 100–200 µg and a half maximal response between 5 and 10 µg (Fig. 4A and B). The responses were brisk for doses below 1 µg, slightly increased their duration in the 2–20 µg range, further increasing their duration to almost 120 s for doses over 100 µg (Fig. 4C). The mean increases in activity (Δf_{CN}) for the ten tested ganglia are depicted in Fig. 5A. The Δf_{CN} evoked in ganglia from rabbits exposed to CNH presented no statistical significant differences ($P > 0.05$, two-way ANOVA) with those evoked in naïve rabbits. The mean duration of the responses induced by ATP ($n = 8$) were significantly correlated ($r = 0.99$, $P < 0.05$ Student's t-test) with the applied dose (Fig. 5B), with a maximal duration of 130.77 ± 12.13 s, an ED_{50} of 105.36 ± 31.34 µg, and a slope factor of 0.86 ± 0.12. The mean duration curve of the rabbits exposed to CNH was significantly different ($P < 0.05$, two-way ANOVA) from the one of naïve ones, but without significant differences ($P > 0.05$, Fisher F-test) in the slope factor or the ED_{50}. Finally, the responses evoked by successive applications of ATP (every 1 min or more) presented no temporal desensitization in CNH ganglia.

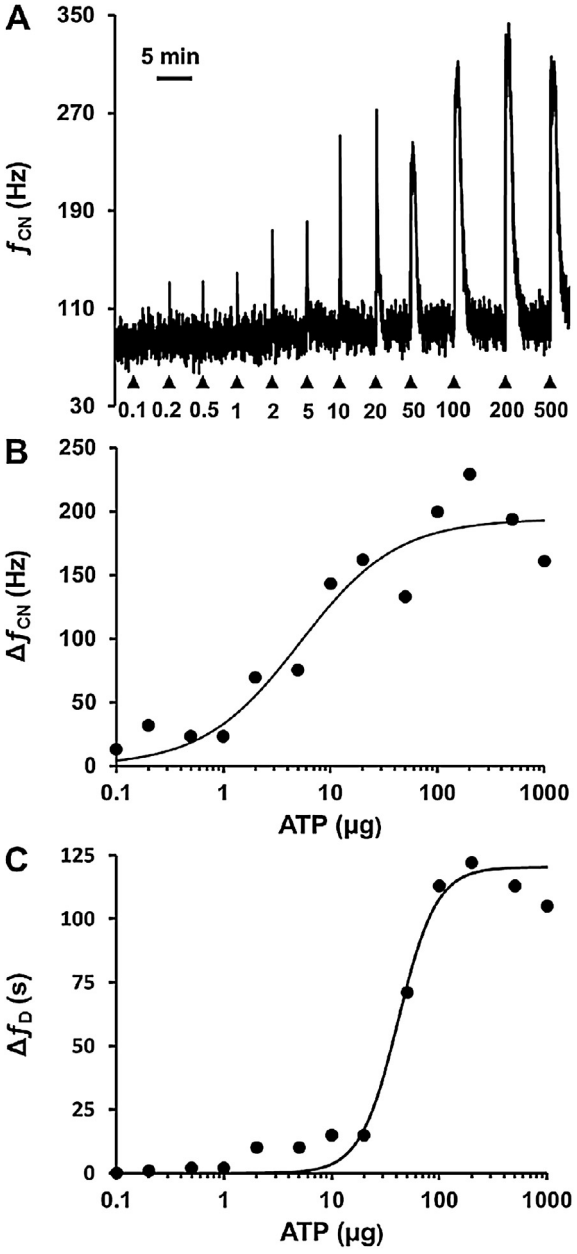
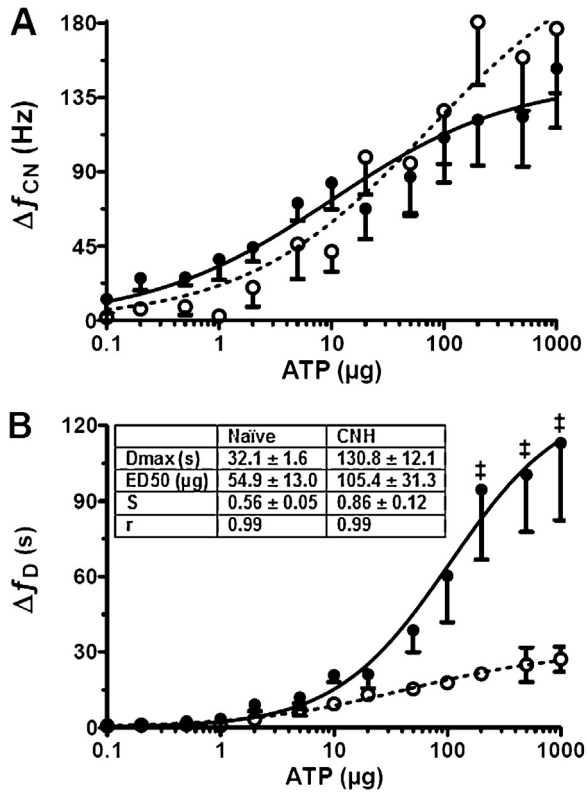


Fig. 4. Responses induced by ATP in a single petrosal ganglion preparation of a rabbit exposed to CNH. (A) Increases in carotid nerve frequency discharge (f_{CN}) evoked by increasing doses of ATP (arrowhead). (B) Dose-response relationship for the increases in the frequency of discharge (Δf_{CN}) in the same preparation. (C) Relationship between the dose and the duration of the responses (Δf_D) induced by ATP in the same preparation.



	Naïve	CNH
Dmax (s)	32.1 ± 1.6	130.8 ± 12.1
ED50 (μg)	54.9 ± 13.0	105.4 ± 31.3
S	0.56 ± 0.05	0.86 ± 0.12
r	0.99	0.99

Fig. 5. Mean effect of ATP on carotid nerve activity in naïve and CNH rabbits. (A) Dose-responses relationship for the mean increases of frequency discharge (Δf_{CN}) show no significant differences between the responses of naïve ($n = 5$) and CNH ($n = 8$) rabbits ($P > 0.05$; two-way ANOVA). (B) Relationship between the dose and the duration (Δf_D) of the ATP-induced responses. The curves were significant different between naïve ($n = 5$) and CNH ($n = 6$) rabbits ($P < 0.01$; two-way ANOVA; ‡$P < 0.001$ Bonferroni test). Inset, table with parameters for the fitted curves; Naïve rabbits, empty circles, dotted line; CNH rabbits, filled circles, continuous line. Data from naïve animals obtained from Soto et al., 2010.

4. Discussion

4.1. Normobaric chronic hypoxia paradigm

Initial reduction of F_{IO_2} was attained by N_2 injection into the chamber, with a steady-state range between 8.2% and 10.4%, and a mean value of 9.07%. However, because the mean relative humidity was higher (≈94%) within the chamber, the actual P_{O_2} value (~64 mm Hg) was below that expected in standard temperature pressure dry (STPD) conditions. Similar hypoxic levels had been used to study physiological modifications induced by CNH (Mosqueira et al., 2012; Schwenke et al., 2008). The F_{IO_2} was maintained low by gas injections, increasing the mean pressure slightly but significantly inside the chamber over the environmental barometric value. Thus, our experiments were performed at a mean barometric pressure of 718.5 mm Hg, equivalent to about 470 m over the sea level. Therefore, the hypoxic exposure was not accompanied by a concomitant decrease in barometric pressure that may generate different physiological responses (Richard and Koehle, 2012; Savourey et al., 2003). It has been reported that ventilatory responses to short term hypoxia appear to differ between hypobaric and normobaric conditions (Savourey et al., 2003). Moreover, in contrast to chronic hypobaric hypoxia in rats, where ventilation increases after 14 days (Olson and Dempsey, 1978), the basal ventilation is slightly depressed after 14 days in rabbits subjected to CNH (Alcayaga et al., 2012).

4.2. Growth and hematocrit modifications by CNH

Body weight remained almost unchanged during the 14 day CNH period. However, according to rabbit growth curves (Blasco et al., 2003; Larzul and De Rochambeau, 2004), the expected weight was 2.42 ± 0.05 kg after the CNH period, significantly heavier ($P < 0.01$; Student's paired t-test) than the final weight of rabbits exposed to CNH. Under a similar CNH paradigm animals present a slight decrease in weight (Mosqueira et al., 2012; Neckář et al., 2003), with a fast recovery after returning to normoxia (Mosqueira et al., 2012). Thus, the CNH period appear no to permanently modify the metabolism of adult animals as it has been reported for rats exposed to hypobaric hypoxia during postnatal life (Ross et al., 2010). Lack of weight gain and pulmonary hypertension has been

associated with acute and chronic hypoxia (Levine et al., 1988). Hypoxia increases pulmonary pressure but only hypobaric hypoxia increases left atrial pressure, and lung fluid and protein flow (Levine et al., 1988). Thus, pulmonary edema is not expected to develop in the animals exposed to CNH and is unlikely to underlie the observed weight stagnation during the CNH exposure.

The exposure to CNH increased hematocrit from a mean basal level of 40.5% to a value of 54.6%. The hematocrit measured before the CNH exposure was similar to that previously described for rabbits living in large confined spaces (Harcourt-Brown and Baker, 2001). Similar or even larger modifications of the hematocrit have been reported in rats (Neckář et al., 2003) and mice (Mosqueira et al., 2012) exposed to CNH. Rabbits presented similar hematocrit increases after 24 days of exposure to chronic hypobaric intermittent hypoxia of similar magnitude ($P_{O_2} \approx 67$ mm Hg; Jelkmann et al., 1979). The increase in hematocrit is expected as a result of increased erythropoietin levels (Prabhakar and Semenza, 2012) at a constant plasma volume (Jelkmann et al., 1979) observed in chronic hypoxic challenges.

4.3. Acetylcholine-induced responses after CNH

Application of ACh to petrosal ganglia exposed to CNH increased f_{CN} in a dose-dependent manner. Similar responses had been reported for petrosal ganglion neurons of several species (Alcayaga et al., 1998, 2007; Soto et al., 2010; Zhang et al., 2000; Zhong and Nurse, 1997). These responses appear to be largely the result of activation of nicotinic receptors (Alcayaga et al., 1998, 2007; Zhang et al., 2000; Zhong and Nurse, 1997), although muscarinic receptors appear to mediate inhibitory responses in the rabbit petrosal ganglion (Soto et al., 2010) and carotid body (Docherty and McQueen, 1979; Monti-Bloch and Eyzaguirre, 1980). Conversely to the actions of muscarinic receptors in petrosal ganglia of naïve animals (Soto et al., 2010), activation of muscarinic receptors with bethanechol was devoid of any noticeable effect on the basal activity or the responses induced by ACh in the ganglia of rabbits exposed to CNH. This result suggests that expression of muscarinic receptors and/or its coupling to neuronal electrical activity is modified by the CNH exposure. There were no significant modifications in the relationship between the ACh dose and the increases in f_{CN} in ganglia from rabbits exposed to CNH with respect to naïve animals. However, in rats exposed to chronic hypobaric hypoxia for 9–22 days the ACh-induced chemosensory responses recorded *in vitro* were potentiated (He et al., 2005). Our responses to maximal ACh doses appear to be slightly increased, although this modification was not statistically significant. The observed differences may arise from the different physiological adaptations that result from hypobaric exposition (Levine et al., 1988; Richard and Koehle, 2012; Savourey et al., 2003). Moreover, in the same rat preparation chemosensory responses to hypoxia and hypercapnia, largely (~80%) blocked in control preparations, were not significantly modified during the application of mecamylamine in chronically hypoxic animals (He et al., 2005). On the other hand, increased ventilatory and chemosensory responses induced by chronic intermittent hypoxia (Rey et al., 2004) are not accompanied by changes in the ACh dose-response relationship of petrosal ganglion neurons (Iturriaga and Alcayaga, 2007). Thus, modifications brought about by chronic hypoxia may affect differentially the chemoreceptor cells and the petrosal ganglion neurons.

Despite of the lack of changes in the dose-response relationship by CNH, the duration of the ACh-induced responses were largely increased after the exposure to CNH. The maximal duration of the dose-duration response was almost doubled in ganglia of CNH rabbits with respect to responses of naïve rabbits. This modification in maximal duration was achieved without a significant change in the sensitivity or the steepness of the relationship. In rats, chronic hypobaric hypoxia enhances ACh-induced responses without noticeable changes in duration (He et al., 2005). However, in naïve rabbit petrosal ganglion responses to ACh are significantly enhanced in amplitude and duration by prior muscarinic receptor blockade with atropine (Soto et al., 2010). In petrosal ganglia of rabbits exposed to CNH a muscarinic receptor agonist had no effect on the basal activity and does not modulate responses induced by ACh, suggesting that the lack of muscarinic response observed in CNH preparations could be partly responsible for the increased duration of the ACh-induced responses. Because petrosal ganglion neurons express several subunits of the nicotinic receptor, such as α3 and α4, and β2 (Shirahata et al., 1998), it is possible that the increased duration observed in CNH preparations may be the result of the modification of the expression and/or content of different heteromeric receptors that could modify the temporal course of the responses. It is noteworthy that ACh-induced responses presented temporal desensitization in ganglia exposed to CNH, while desensitization was absent in naïve animals (Soto et al., 2010), further suggesting a modification of nicotinic ACh receptors induced by CNH. The increased duration of ACh-induced responses may underlie the increased sensibility of the ventilatory reflex induced by intravenous NaCN injections in rabbits exposed to CNH (Alcayaga et al., 2012).

4.4. ATP-induced responses after CNH

Application of ATP to petrosal ganglia exposed to CNH for 14 days increased f_{CN} in a dose-dependent manner, as it has been previously reported for petrosal ganglion neurons of several species (Alcayaga et al., 2000, 2007; Soto et al., 2010; Zhang et al., 2000). The mean dose-response curve of the responses recorded from petrosal ganglia exposed to CNH was not significantly different from that obtained from naïve rabbits (Soto et al., 2010). These responses appear to be largely the result of activation of nucleotide-activated ionotropic (P2X) receptors (Alcayaga et al., 2000, 2007; Zhang et al., 2000; Zhong and Nurse, 1997) that are expressed in petrosal ganglion neurons (Prasad et al., 2001). Chronic hypobaric hypoxia has no effect on the expression of P2X2 subunits in the petrosal ganglion (He et al., 2006). Because we did not use antagonist in these experiments we cannot rule out the involvement of metabotropic nucleotide (P2Y) receptors. However, in naïve animals of several species the responses evoked by ATP are fully dependent on P2X receptors with no apparent contribution of P2Y receptors (Alcayaga et al., 2000, 2007; Soto et al., 2010; Zhang et al., 2000). In rats submitted to chronic hypobaric hypoxia for 9–16 days, where both basal activity and acute hypoxia induced responses are increased with respect to control (normoxic) conditions, the blockade of P2X nucleotide receptors had no effect on basal activity but reduced the increase in chemosensory activity induced by acute hypoxia to a significantly lesser extent in chronically hypoxic than in control rats (He et al., 2006). Thus, the relevance of ATP in chemosensory afferent activity appears to be reduced after chronic hypoxia. This appears not to be the case in our CNH preparations, but our recordings were restricted to the post-synaptic component of the chemoafferent pathway, while application of antagonists to the carotid body may affect both the pre- and post-synaptic elements (He et al., 2006).

Responses to ATP were significantly increased in duration as a result of exposure to CNH. This modification was not accompanied by a change in sensibility (ED_{50}) or the steepness of the relationship. It has been reported that activation of petrosal ganglion neurons *in vitro* induces release -at least- of dopamine (Iturriaga et al., 2003). Chronic hypobaric hypoxia modifies the expression of modulatory peptides in the carotid body (Chen et al., 2002b), acid-sensitive ion channels (ASICs) in the petrosal ganglion (Liu et al., 2011) and connexin 43 in both structures (Chen et al., 2002a). Thus, the activation

of nucleotide receptors in the ganglion may induce release of other transmitter molecules (Iturriaga et al., 2003), through exocytosis or connexin hemichannels (Stout et al., 2002), that can directly and/or indirectly increase the duration of the response. The petrosal ganglion neurons appear to express a heterotrimeric form of the P2X receptor, the P2X2/3 receptor (Alcayaga et al., 2007; Prasad et al., 2001). Nucleotide P2X receptors present a wide range of response duration and desensitization, depending on the subunit composition of the channel (see North, 2002). Thus, the exposure to CNH may modify the expression of different receptor subunits, the final composition of the P2X receptor, and the temporal course of the response. The increased duration may underlie the increased sensibility of the ventilatory reflex induced in CNH animals by intravenous NaCN injections (Alcayaga et al., 2012).

5. Conclusions

In summary, exposure to our model of CNH for 14 days produces an increase in hematocrit and a stagnation of the growth of the animals. The responses induced in the petrosal ganglion by the application of ACh and ATP presented a similar dose-response curve that animals not exposed to hypoxia (naïve). However, the evoked responses were of longer duration and the maximal responses to ACh and ATP were increased by approximately two and four times, respectively, with respect to those elicited in naïve animals. The increased duration may underlie the increased sensibility of the ventilatory reflex observed in rabbits exposed to CNH. Moreover, in contrast to naïve rabbits, responses induced by ACh in CNH ganglia appeared to be devoid of muscarinic components and presented temporal desensitization.

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