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Responses induced by acetylcholine and ATP in the rabbit petrosal ganglion

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ABSTRACT

Acetylcholine and ATP appear to mediate excitatory transmission between receptor (glomus) cells and the petrosal ganglion (PG) neuron terminals in the carotid body. In most species these putative transmitters are excitatory, while inhibitory effects had been reported in the rabbit. We studied the effects of the application of acetylcholine and ATP to the PG on the carotid nerve activity *in vitro*. Acetylcholine and ATP applied to the PG increased the carotid nerve activity in a dose-dependent manner. Acetylcholine-induced responses were mimicked by nicotine, antagonized by hexamethonium, and enhanced by atropine. Bethanechol had no effect on basal activity, but reduced acetylcholine-induced responses. Suramin antagonized ATP-induced responses, and AMP had little effect on the carotid nerve activity. Our results suggest that rabbit PG neurons projecting through the carotid nerve are endowed with nicotinic acetylcholine and purinergic P2 receptors that increase the carotid nerve activity, while simultaneous activation of muscarinic cholinergic receptors reduce the maximal response evoked by nicotinic cholinergic receptor activation.

1. Introduction

The carotid body (CB) is the principal arterial chemoreceptor organ, constituted by specific parenchymal cells: the receptor (glomus, type-I) cells and the glial-like (sustentacular, type-II) cells. The glomus cells are innervated, through the carotid (sinus) nerve, by sensory neurons whose perikarya are located in the petrosal ganglion (PG). The most accepted paradigm of CB chemoreception states that, as a result of the transduction mechanism, the glomus cells release one or more transmitters that, acting on postsynaptic receptors located on the nerve terminals of PG neurons, generate or maintain the afferent activity (Eyzaguirre and Zapata, 1984; González et al., 1994; Prabhakar, 2000). There are several transmitter molecules present in the CB, but their exact participation in the generation of the chemo-afferent activity is still controversial. Part of the controversy arises from conflicting results obtained from different species and preparations.

Acetylcholine (ACh) was one of the first molecules postulated as a transmitter in the CB (see Heymans, 1955). ACh and its metabolic synthesis and degradation pathways are present in the CB of cats (Fidone et al., 1976; Wang et al., 1989), rabbits (Wang et al., 1989; Kim et al., 2004) and in the rat CB-PG reconstituted system *in vitro* (Nurse and Zhang, 1999). ACh is released from the cat CB during electrical (Eyzaguirre and Zapata, 1968) and hypoxic stimulation (Fitzgerald et al., 1999; Fitzgerald, 2000), but ACh release from the rabbit CB is reduced during hypoxic stimulation (Kim et al., 2004). The exogenous application of ACh to the cat CB increases the carotid nerve frequency of discharge (Eyzaguirre and Zapata, 1968), while its effect is mainly inhibitory in the rabbit CB (Docherty and McQueen, 1979; Monti-Bloch and Eyzaguirre, 1980). However, application of nicotine to the CB increases the carotid nerve frequency of discharge both in the cat (Eyzaguirre and Zapata, 1968; Reyes et al., 2007) and in the rabbit (Monti-Bloch and Eyzaguirre, 1980; Jonsson et al., 2004), effects blocked by nicotinic ACh receptor antagonists (Eyzaguirre and Zapata, 1968; Jonsson et al., 2004; Reyes et al., 2007). On the other hand, muscarinic ACh receptor subtypes M1 and M2 have been described in the cat CB (Shirahata et al., 2004). However, muscarinic antagonists have no effect on the cat chemosensory activity but depressed the carotid nerve frequency of discharge on the rabbit (Monti-Bloch and Eyzaguirre, 1980). In the rabbit CB, atropine antagonizes the inhibitory effects of muscarinic ACh receptor agonists on the carotid nerve frequency of discharge (Monti-Bloch and Eyzaguirre, 1980), increases basal ACh release and revert the reduction of ACh release induced by hypoxia to an increased release (Kim et al., 2004). Thus, the available data indicate that ACh appear to be excitatory in the cat CB, but may have both excitatory and inhibitory actions in the rabbit, mediated by nicotinic and muscarinic ACh receptors, respectively. However, the exogenous application of ACh to the CB may involve activation

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of both presynaptic and postsynaptic receptors (Kim et al., 2004; Shirahata et al., 2007).

Currently, is accepted that both ATP and adenosine (Buttigieg and Nurse, 2004; Conde and Monteiro, 2004, 2006a) are released from the rat CB by hypoxia (see Conde and Monteiro, 2006b). ATP increases the carotid nerve frequency of discharge when applied to the rat CB in situ and *in vitro* (McQueen and Ribeiro, 1981; Spergel and Lahiri, 1993), although the action of ATP through its metabolites AMP (McQueen and Ribeiro, 1983) or adenosine (Runold et al., 1990) has been suggested. In the rat CB preparation *in vitro*, adenosine appears to participate in the response to an hypoxic challenge, acting both on PG and CB cells trough A2A and A2B receptors, respectively (Conde et al., 2006). On the other hand, using the cat PG preparation *in vitro* we found that the application of ATP increases the carotid nerve frequency of discharge in a dose-dependent manner, effect that is only marginally mimicked by AMP (Alcayaga et al., 2000a,b). These data suggest that ATP increases the chemosensory activity in the rat and cat at the postsynaptic level in the PG neurons, but there is no information on the effects of ATP on the rabbit carotid chemosensory afferents.

Both ACh and ATP have been proposed to participate in the generation of the afferent chemosensory activity by acting on specific receptors located on the PG terminals in the CB of cats and rats (Prasad et al., 2001; Iturriaga and Alcayaga, 2004; Nurse, 2005). However, in the rabbit the effect of ACh is controversial and little or no information about the effect of ATP is available (see Alcayaga et al., 2006; Iturriaga et al., 2007). Thus, we studied the responses evoked by ACh and ATP on the rabbit PG neurons that project through the carotid nerve, using an isolated PG preparation (Alcayaga et al., 1998).

2. Methods

2.1. Animals

Experiments were performed on 24 male adult White New Zealand rabbits (2.30 ± 0.11 kg; mean ± SEM). The protocol was approved by the Ethical Committee of the Facultad de Ciencias of the Universidad de Chile, and meets the guidelines of the National Fund for Scientific and Technological Research (FONDECYT), Chile, and the Guiding Principles for the Care and Use of Animals of the American Physiological Society.

2.2. Surgical procedures and recording of nerve activity

The PGs were obtained from animals anaesthetized with an intramuscular injection of a mixture of ketamine (75 mg/kg) and xylazine (7.5 mg/kg). Additional intramuscular doses (1/3 of the initial dose) were applied when necessary to maintain the surgical anesthetic level. The carotid bifurcation was exposed through an incision in the neck midline, and the carotid nerve was cut close to the CB. The glossopharyngeal nerve, severed peripherally to the apparent origin of the carotid nerve, was followed into the skull eroding the tympanic bulla and the petrosal bone to expose the PG. The glossopharyngeal nerve was severed cephalically to the central apparent limit of the ganglion. The tissue thus obtained was placed in ice-chilled Hanks' solution, the capsule removed from over the ganglion, and the epineurium was excised along the full extension of the nerves. The ganglion was pinned to the bottom of a 0.2 mL chamber, over a pair of stimulating electrodes, and superfused with air-equilibrated Hanks' buffered salt solution (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 lifted into the upper compartment of the superfusion chamber filled with mineral oil. The electrodes were connected in turn to an AC-preamplifier, and the resulting electroneurogram was amplified, displayed in oscilloscope, and recorded on a videocassette recorder after digital encoding. The electroneurogram was also fed to a spike amplitude discriminator, whose standardized pulses were digitally counted, to assess the carotid nerve frequency of discharge (f_{CN}), in Hz. The temperature of the chamber and the f_{CN} were acquired, displayed and recorded through a PC-based custom-made data acquisition system, at 1 Hz. ACh, nicotine, bethanechol, and ATP, in doses of 1–3000 μg in 10 μL boluses, were applied over the ganglion. Antagonists were supplied at constant concentration in the superfusion medium for at least 40 min before its effects were evaluated.

2.3. Electrical stimulation of the petrosal ganglion

The functional integrity of the PG preparation was evaluated at the beginning and end of each experiment by applying brief electrical pulses (50–70 μs) to the ganglion and recording the compound action potentials in the carotid nerve and the glossopharyngeal branch. Only preparations that showed no major decrease in the amplitude of the compound action potential were included in the analyses. The conduction velocities were estimated using the distance between the stimulating and recording electrodes, and the conduction time assessed from the stimulus artifact to the maximum of each wave.

2.4. Data evaluation and statistical analysis

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 activity, computed in a 30 s interval prior to an evoked response. The relation between Δf_{CN}s and the doses of any of the drugs used (X) was assessed by fitting the standardized responses $\Delta f/\Delta f_{max} = (\Delta f_{CN}/\max \Delta f_{CN})$ to a sigmoid curve ($[\Delta f/\Delta f_{max}] = 1/[1 + \{ED_{50}/D\}^S]$), where D = applied dose, ED_{50} = the dose that evoked half-maximal response, and S = Hill slope factor determining the steepness of each curve. Significance of correlation between variables was assessed using Student's t-test. Differences between related samples were assessed using Student paired t-test or repeated measures two-way ANOVA, depending on the data structure, and the significance level set at $P < 0.05$. All data presented as mean ± SEM. All curve fitting and statistical calculations were performed using GraphPad Prism software (GraphPad Software, USA).

3. Results

3.1. Electrical stimulation

Electrical stimulation of the PG elicited an antidromic compound action potential in both the carotid nerve and the glossopharyngeal branch of the glossopharyngeal nerve. Fig. 1 shows representative compound action potentials recorded from the peripheral branches of the glossopharyngeal nerve. The compound action potential recorded in the glossopharyngeal branch was dominated by components attributable to fast conducting fibers (Fig. 1A), while the compound action potential evoked in the carotid nerve was dominated by slower conducting components (Fig. 1B). The fastest components of the carotid nerve compound action potential comprised components with conduction velocities faster than 2.0 m/s, velocities corresponding to myelinated fibers—according to Erlanger and Gasser's classification—while slowest conducting components (conduction velocity <1.5 m/s) were attributed to unmyelinated fibers (Fig. 1B). Conversely, the largest components of the glossopharyngeal branch compound

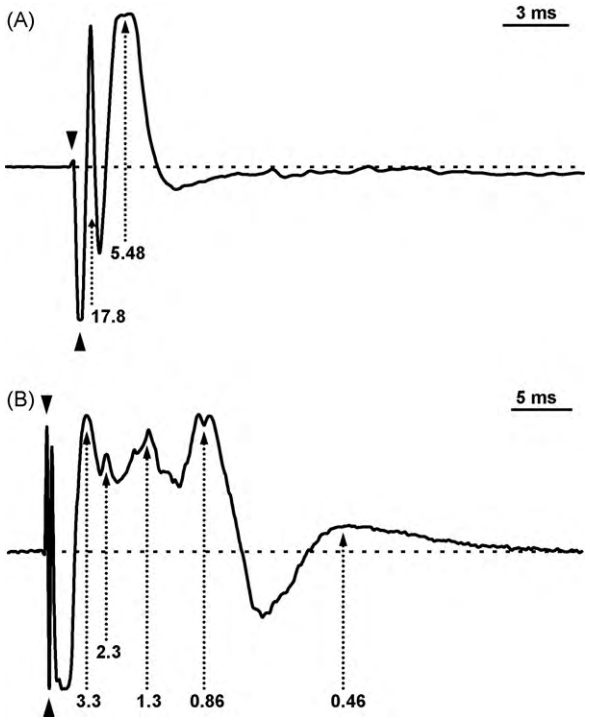

Fig. 1. Compound action potentials recorded from the branches of the rabbit glossopharyngeal nerve. Average of 12 consecutive responses elicited by 70 μs square electrical pulses delivered every second to the petrosal ganglion. (A) Recording from the glossopharyngeal branch. (B) Recording from the carotid nerve. Numbers and arrows indicate the estimated conduction velocity, in m/s, for each component of the compound action potential. Arrowheads indicate stimulus artifact.

action potential presented conduction velocities faster than 5 m/s (Fig. 1A)

3.2. Acetylcholine

The application of ACh to the PG produced a fast increase of f_{CN}, which amplitude and duration increased with the dose. Fig. 2 depicts the increases in f_{CN} induced by increasing doses of ACh, applied every 5 min, in a single preparation. In this preparation, the increases in f_{CN} (Δf_{CN}) induced by ACh presented a threshold of about 1 μg, the amplitude of the response increased with increasing doses, and attained a plateau of maximal activity for doses over 50 μg (Fig. 2A and B). Similarly, the duration of the ACh-induced response, measured as the period where f_{CN} remained significantly increased over the confidence limit (99%) of the mean basal f_{CN}, increased in a dose-dependent manner, attaining a plateau for doses larger than 50 μg (Fig. 2C). The Δf_{CN} evoked by each ACh dose were standardized to the maximal response evoked by ACh on each experiment. The standardized mean dose-Δf_{CN} relationship shows that the variables were highly ($r = 0.99$; $n = 13$) and significantly ($P < 0.001$; Student's t-test) correlated, with a mean ED_{50} of 8.15 ± 1.21 μg, and a slope of 0.67 ± 0.06 (Fig. 3A). Similarly, the mean dose–duration relationship showed a high and significant correlation ($r = 0.99$; $P < 0.001$; Student's t-test; $n = 7$), with a maximal duration of 24.29 ± 0.92 s, an ED_{50} of 7.33 ± 1.25 μg and a slope factor of 0.92 ± 0.12 (Fig. 3B).

The responses induced by ACh presented little or no temporal desensitization. Fig. 4A shows that 4 maximal doses of ACh (1000 μg) induced almost the same increases in f_{CN}, although the doses were delivered at 1 min intervals. Bethanechol, a muscarinic agonist not degraded by acetylcholinesterase, was ineffective in modifying f_{CN} even at doses as high as 1000 μg (Fig. 4A). However, the responses induced by the same maximal dose of ACh were

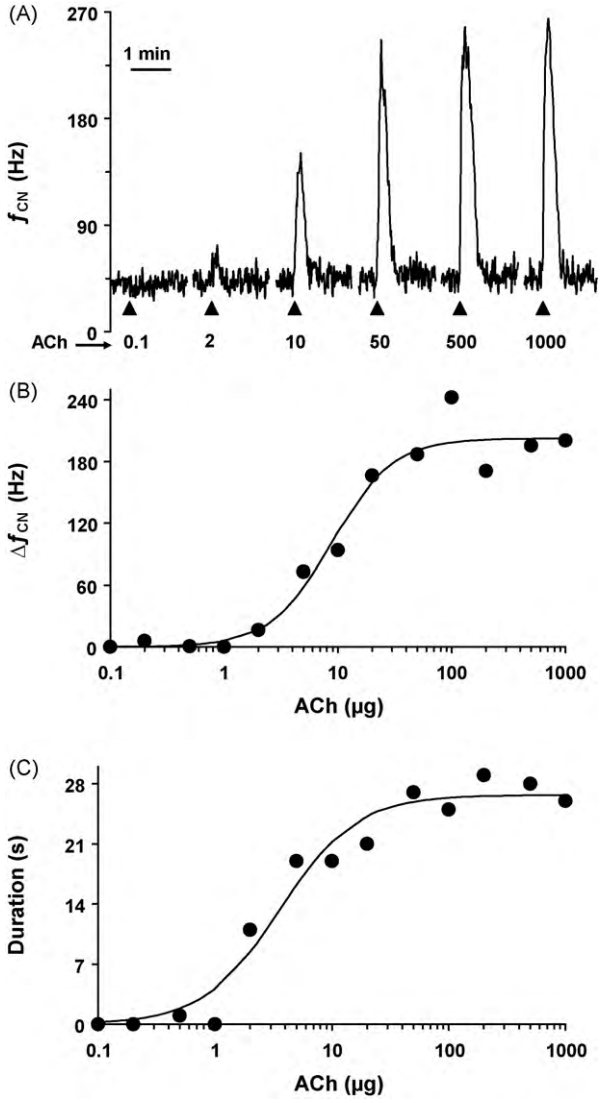

Fig. 2. Effect of ACh on the carotid nerve activity in a single preparation. (A) Increasing doses of ACh (arrowheads) increases carotid nerve frequency of discharge (f_{CN}). (B) Relationship between ACh dose and the increase in carotid nerve frequency of discharge (Δf_{CN}). (C) Dose–duration relationship.

reduced in amplitude during a 4–5 min interval after the application of bethanecol (Fig. 4A). Fig. 4B depicts the mean response of four consecutive responses induced by ACh (1000 μg) recorded prior to (control) and after the application of bethanechol (500 μg), showing a significant reduction ($P = 0.017$; Student's paired test) of the responses induced by ACh after the activation of muscarinic receptors. The inclusion of 10 μM atropine, a muscarinic receptor blocker, in the superfusion medium in three preparations produced a significant increase in the magnitude of the ACh-induced responses ($P < 0.001$; two-way ANOVA), without a significant modification the sensitivity (ED_{50}; control = 2.00 ± 0.30 vs. atropine = 2.49 ± 0.33) or the slope (S; control = 2.16 ± 0.62 vs. atropine = 1.84 ± 0.36) of the dose-amplitude relationship ($P > 0.05$; two-way ANOVA) (Fig. 5A). Similarly, in the same preparations the duration of the ACh-induced responses were significantly increased ($P < 0.001$; two-way ANOVA) during atropine superfusion, without major modification of the sensitivity (ED_{50}; control = 2.97 ± 0.69 vs. atropine = 2.53 ± 0.31) or the slope (S; control = 1.10 ± 0.24 vs. atropine = 1.31 ± 0.17) of the dose–duration relationship ($P > 0.05$; two-way ANOVA) (Fig. 5B).

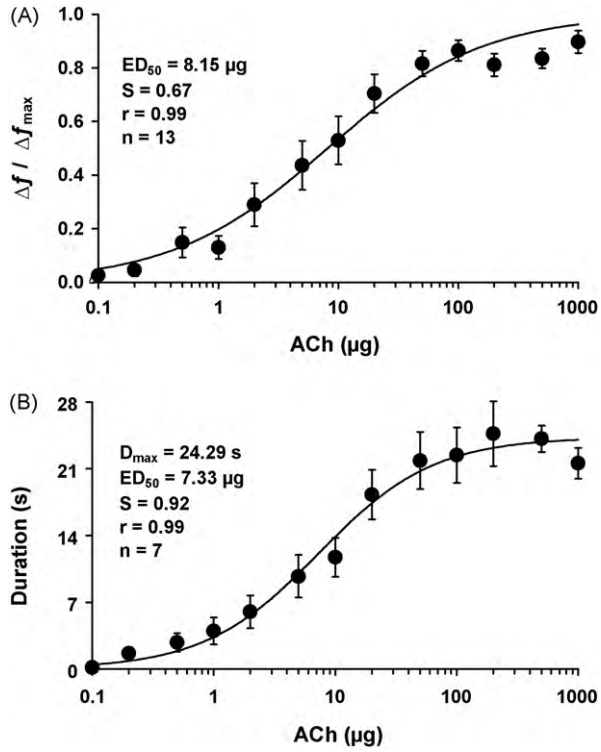

Fig. 3. Mean effect of ACh on the rabbit carotid nerve activity. (A) Relationship between ACh dose and the standardized increases in carotid nerve frequency of discharge ($\Delta f/\Delta f_{max}$). (B) Dose–duration relationship.

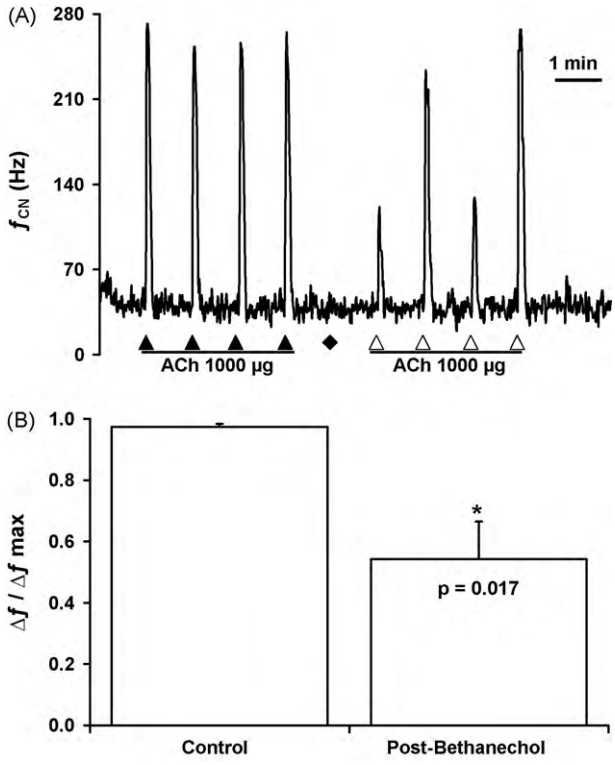

Fig. 4. Effect of bethanechol on the ACh-induced responses in the carotid nerve. (A) Applications of ACh (filled arrowheads) every 60 s increased the carotid nerve frequency of discharge (f_{CN}). Bethanechol (filled diamond; 1000 µg) had no apparent effect on f_{CN}, but reduced the amplitude of ACh-induced responses (empty arrowheads) during the following 4 min. (B) The mean standardized increases in carotid nerve frequency of discharge ($\Delta f/\Delta f_{max}$) evoked by 4 successive ACh applications in control conditions were significantly reduced ($P<0.05$; paired Student t-test) by bethanechol (1000 µg).

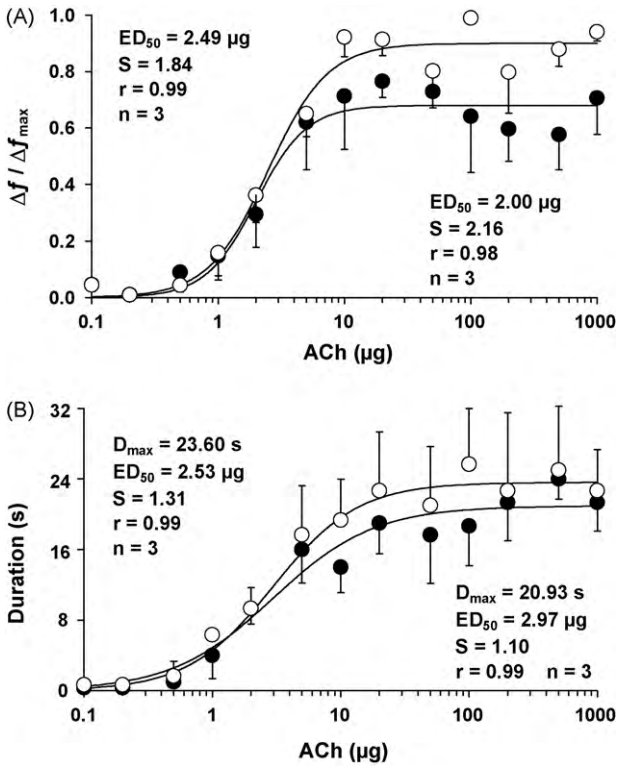

Fig. 5. Effect of atropine on the responses evoked by ACh in the carotid nerve. (A) The mean standardized increases in carotid nerve frequency of discharge ($\Delta f/\Delta f_{max}$) evoked in control conditions (filled circles) were significantly increased in amplitude ($P<0.05$; two-way ANOVA) during atropine superfusion (empty circles), without significant modification of the sensitivity (ED_{50}) or the slope (S) of the dose–response relationship ($P>0.05$; two-way ANOVA). (B) The mean duration of the responses evoked in control conditions (filled circles) were significantly increased ($P<0.05$; two-way ANOVA) during atropine superfusion (empty circles), without significant modification of the sensitivity or the slope of the dose–response relationship ($P>0.05$; two-way ANOVA).

Application of nicotine (0.1–1000 µg) increased f_{CN} in a dose-dependent manner, but the evoked responses presented a high degree of temporal desensitization (not shown). On the other hand, the responses induced by ACh were blocked when the preparation was superfused with HBSS containing 10 µM hexamethonium. Fig. 6A shows the ACh-induced responses in a single preparation, its reduction by near 66% during the superfusion with hexamethonium (10 µM), and the complete recovery of the responses after the exclusion of hexamethonium from the superfusion medium. The mean effect of hexamethonium on the ACh-induced responses on three preparations is showed in Fig. 6B. Hexamethonium produced a significant reduction of the ACh-induced responses ($P<0.001$; two-way ANOVA), without significant modification the sensitivity (ED_{50}; control = 7.17 ± 1.17 vs. hexamethonium = 12.44 ± 3.62) or the slope (S; control = 1.81 ± 0.57 vs. hexamethonium = 1.75 ± 0.74) of the dose-amplitude relationship ($P>0.05$; two-way ANOVA) (Fig. 6B).

3.3. Adenosine 5′-triphosphate

ATP applied to the PG produced fast increase of f_{CN}, which amplitude and duration increased in a dose-dependent manner, without any noticeable temporal desensitization of the responses. Fig. 7A shows the responses induced by increasing ATP doses, applied every 5 min, in a single preparation. The Δf_{CN}s induced by ATP were detectable for doses of about 2 µg, the amplitude of the response continued increasing with increasing doses, attaining the maximal response for the largest dose tested. Similarly, the

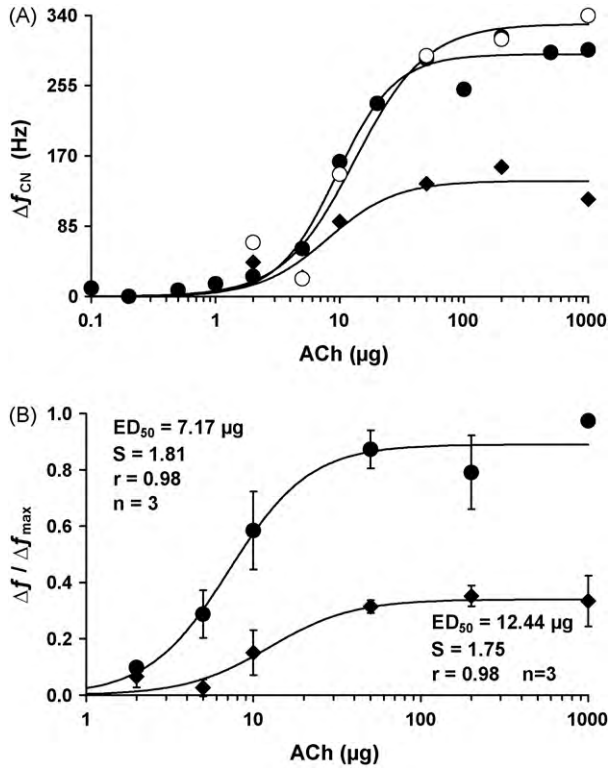

Fig. 6. Effect of hexamethonium on the responses evoked by ACh in the carotid nerve. (A) Increases in carotid nerve frequency of discharge (Δf_{CN}) evoked by ACh in control conditions (filled circles), during hexamethonium (filled diamonds), and after (empty circles) blocker washout in a single preparation. (B) The mean standardized increases in carotid nerve frequency of discharge ($\Delta f/\Delta f_{max}$) evoked in control conditions (filled circles) were significantly reduced ($P<0.05$; two-way ANOVA) in amplitude during hexamethonium superfusion (filled diamonds), without significant modification of the sensitivity (ED_{50}) and slope (S) of the dose–response relationship.

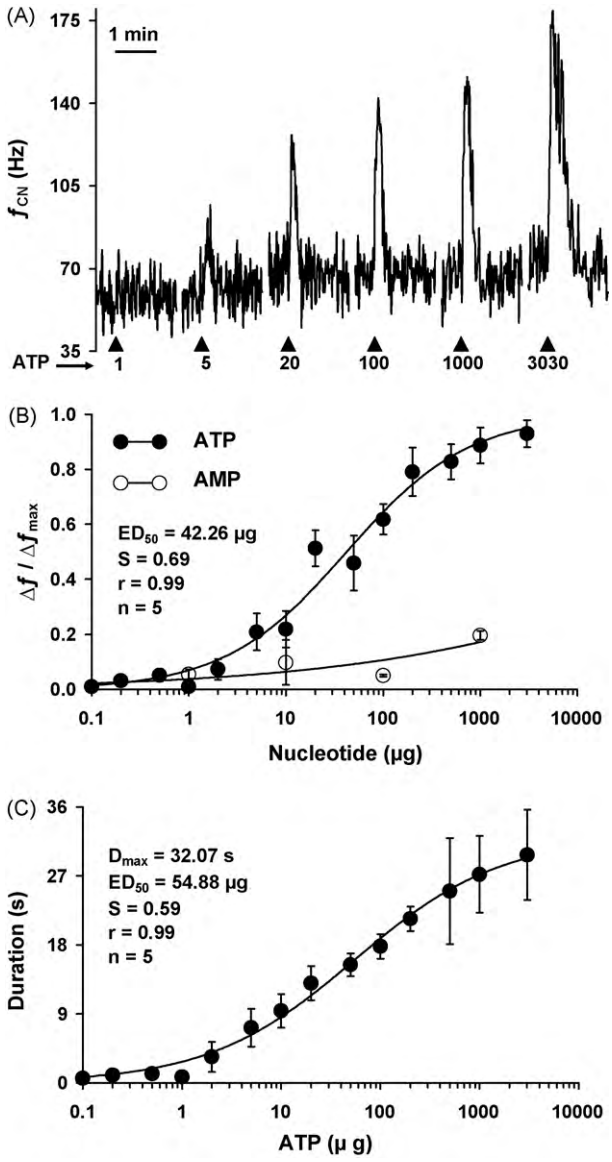

Fig. 7. Effect of ATP on the rabbit carotid nerve activity. (A) Increasing doses of ATP (arrowheads) increases carotid nerve frequency of discharge (f_{CN}) in a single preparation. (B) Mean relationship between ATP (filled circles) and AMP (empty circles) doses and the standardized increases in carotid nerve frequency of discharge ($\Delta f/\Delta f_{max}$). (C) Mean dose–duration relationship for ATP doses.

duration of the response presented a similar threshold, increasing over the whole dose-range. The mean dose–response standardized Δf_{CN} relationship, showed that the variables were highly and significantly correlated ($r=0.99$; $P<0.001$; Student's t-test; $n=5$), with an ED_{50} of 42.26 ± 6.03 μg, and a slope of 0.69 ± 0.06 (Fig. 7B). Similarly, the mean dose–duration relationship showed a high and significant correlation ($r=0.99$; $P<0.001$; Student's t-test; $n=5$), with an ED_{50} of 54.88 ± 12.95 μg, a slope factor of 0.59 ± 0.05, and a maximal duration of 32.07 ± 1.56 s (Fig. 7C). It is noteworthy that AMP was highly ineffective in modifying f_{CN}. Fig. 7B shows the standardized mean increases induced by AMP in two preparations, where the responses induced by AMP attained at the most only 20% of those induced by ATP in the same preparations.

The responses evoked by ATP were antagonized by the addition of suramin (50 μM) to the superfusion medium. Fig. 8A and B shows the effects of suramin on the responses evoked by ATP on two different experiments. During control conditions ATP induced a dose-dependent increase in f_{CN}, while during the superfusion with suramin the responses were completely abolished (Fig. 8A) or highly reduced in sensitivity (Fig. 8B). The mean dose–response standardized Δf_{CN} relationship for five preparations (Fig. 8C) shows that suramin produced a significant reduction in the evoked responses ($P<0.001$; two-way ANOVA) without a significant reduction of sensitivity (ED_{50}; control = 49.59 ± 16.91 vs. suramin = 145.80 ± 140.60) or the slope (S; control = 0.73 ± 0.12 vs. suramin = 0.56 ± 0.15) of the dose-amplitude relationship.

4. Discussion

4.1. General

One main finding of this study is that application of ACh to the rabbit PG increases carotid nerve frequency of discharge, effect blocked by hexamethonium (10 μM) and mimicked by nicotine (0.1–1000 μg) but not by bethanechol (0.1–1000 μg). Acetylcholine-induced responses were transiently reduced by prior application of bethanechol (500–1000 μg), but were enhanced during atropine (10 μM) treatment. Similarly, ATP increases carotid nerve frequency of discharge, responses that were reversibly blocked by suramin (50 μM), while AMP was less effective on increasing the carotid nerve activity. Thus, present results suggest that rabbit PG neurons projecting through the carotid nerve are endowed with nicotinic acetylcholine and purinergic P2 receptors, which increase the carotid nerve frequency of discharge. On the other hand, activation of muscarinic acetylcholine receptors has no

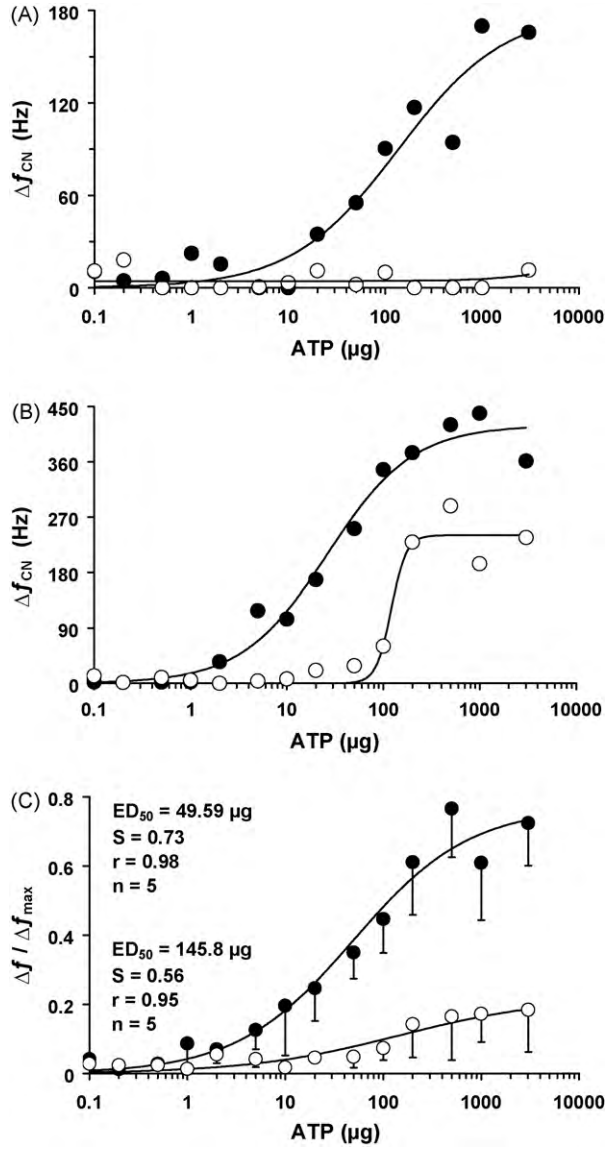

Fig. 8. Effect of suramin on the responses evoked by ATP in the carotid nerve. (A) Increases in carotid nerve frequency of discharge (Δf_{CN}) evoked by ATP in control conditions (filled circles) and during (empty circles) suramin superfusion in a single preparation. (B) Responses evoked in control conditions (filled circles) and during suramin superfusion (empty circles) in a single preparation. (C) The mean standardized increases in carotid nerve frequency of discharge ($\Delta f/\Delta f_{max}$) evoked in control conditions (filled circles) were significantly reduced ($P < 0.05$; two-way ANOVA) in amplitude during suramin superfusion (empty circles).

apparent effect on the basal ongoing activity but appear to reduce the maximal response evoked by nicotinic acetylcholine receptor activation.

4.2. Carotid nerve responses to electrical stimulation

The compound action potentials recorded from the rabbit carotid nerve and the glossopharyngeal branch, induced by electrical stimulation of the PG, indicate that the fibers projecting to the carotid bifurcation are mainly slow conducting fibers. Recordings of the chemosensory activity in the rat (Donnelly, 1999), the mouse (Donnelly and Rigual, 2000), and the cat (Sato et al., 1968) indicate a preponderance of slow conducting fibers in the carotid nerve, while morphological studies of the fiber content in the rat (McDonald, 1983) and the cat (Eyzaguirre and Uchizono, 1961) indicate that small diameter fibers are common in the carotid nerve, although with large differences in their relative numbers on different species (Eyzaguirre and Uchizono, 1961; McDonald, 1983; Donnelly, 1999).

4.3. Acetylcholine-induced responses

The present data indicate that rabbit PG neurons projecting through the carotid nerve increase their frequency of discharge in response to applications of ACh to their soma. This response increased in a dose-dependent manner, both in amplitude and in duration. Similar responses to ACh have been described in the cat PG *in vitro* (Alcayaga et al., 1998), in cat PG neurons in tissue culture (Varas et al., 2000; Shirahata et al., 2000; Alcayaga et al., 2003; Varas et al., 2006), and in rat jugular-petrosal ganglion neurons alone or co-cultured with CB tissue (Zhong and Nurse, 1997; Zhong et al., 1997; Nurse and Zhang, 1999). The responses evoked in the rabbit were mimicked by nicotine and largely and reversibly blocked by hexamethonium (10 µM), suggesting that nicotinic cholinergic receptors mediate most of these responses. Similar pharmacological properties have been described for the receptors involved in the ACh-induced increase of frequency of discharge in the cat carotid nerve *in vivo* (Reyes et al., 2007), PG *in vitro* (Alcayaga et al., 1998) and in the responses evoked by ACh in isolated neurons of the cat (Alcayaga et al., 2003; Varas et al., 2006) and rat jugular-petrosal ganglion (Zhong and Nurse, 1997; Koga and Bradley, 2000).

In the cat PG the ACh-induced responses presented a high degree of temporal desensitization (Alcayaga et al., 1998). However, the responses evoked by ACh in the rabbit presented little or no temporal desensitization, and the duration of the responses were dose-dependent. These differences suggest that the nicotinic receptors expressed by PG neurons (Varas et al., 2006) may differ between species. The application of a muscarinic cholinergic agonist to the rabbit PG *in vitro* had no effect on the basal ongoing activity, but reduced the responses evoked by further applications of ACh to the ganglion. Moreover, both the amplitude and the duration of the responses induced by ACh were enhanced in the presence of atropine, a muscarinic cholinergic receptor blocker. It has been described that ACh has an inhibitory effect on the rabbit afferent carotid chemosensory activity when applied to the CB (Docherty and McQueen, 1979; Monti-Bloch and Eyzaguirre, 1980). In cat PG neurons in culture voltage clamp recordings show that ACh induces a nicotinic receptor-mediated inward current (Alcayaga et al., 2007; Varas et al., 2006), but an outward current has also been reported (Shirahata et al., 2000). Thus, existence of ionotropic and metabotropic ACh receptors in PG neuron terminals may explain this opposite effects.

Muscarinic ACh receptors have been reported to be present in rabbit CB parenchymal cells, but absent in the PG nerve terminals (Dinger et al., 1986, 1991), while nicotinic ACh receptors are present in PG terminals (Shirahata et al., 1998) and in cultured glomus cells of cats (Higashi et al., 2003). Our results suggest that rabbit PG neurons appear to be endowed with both muscarinic and nicotinic cholinergic receptors that are simultaneously activated by the exogenous application of ACh. Nicotinic cholinergic receptors mediate the increase in frequency of discharge, while muscarinic cholinergic receptors appear to partially inhibit the excitatory response mediated by cholinergic receptors. A similar expression of nicotinic and muscarinic receptors with excitatory and inhibitory actions, respectively, has been reported in rat dorsal root ganglion neurons (Genzen et al., 2001; Dubé et al., 2005; Hayashida et al., 2006). Nevertheless, because the recorded responses arise from a large population of neurons, we cannot rule out the possibility that a small population of PG neurons express muscarinic receptors which activation leads to neuronal excitation.

4.4. ATP-induced responses

In this study we confirm and extend previous reports on ATP effects on rabbit PG neurons (Alcayaga et al., 2006; Iturriaga et al., 2007). Indeed, we found that the application of ATP to the rabbit PG increased the frequency of discharge in the carotid nerve in a dose-dependent manner. The duration of the response presented a similar dose dependency. Our previous work indicated that application of ATP to the cat PG *in vitro* increases the frequency of discharge from the carotid nerve (Alcayaga et al., 2000a,b). Similarly, cat PG neurons (Alcayaga et al., 2003; Varas et al., 2003) and neurons from the rat jugular-petrosal complex (Zhang et al., 2000) are depolarized by ATP, that induces inwardly directed currents at resting membrane potential (Zhang et al., 2000; Alcayaga et al., 2003, 2007). Intracellular recordings from rat jugular-petrosal neurons in co-culture with CB tissue show that increased neuronal activity evoked by chemosensory stimuli are partially blocked by suramin (Zhang et al., 2000). Similarly, recordings from cat PG neurons in an acutely isolated preparation, in which the CB and PG remain functionally connected, indicate that responses to acidification and stop-flow are partially blocked by suramin (Varas et al., 2003). Those same identified cat petrosal chemosensory neurons responded to somatic application of ATP with depolarization and increased firing rate (Varas et al., 2003), indicating that PG chemosensory neurons express ATP receptors both at their soma and terminals. Immunohistological evidence shows that $P2X_2$ and $P2X_3$ receptor subunits are expressed in the rat CB and jugular-petrosal neurons (Prasad et al., 2001). Similarly, both mRNA and protein of $P2X_2$ and $P2X_3$ receptor subunits are present in cat PG neurons (Bairam et al., 2007). Moreover, selective deletion of genes encoding for $P2X_2$ and $P2X_3$ receptor subunits in mice indicate that afferent chemosensory responses to hypoxia are largely reduced in the absence of $P2X_2$ subunit, but are further reduced if $P2X_3$ subunit is deleted (Rong et al., 2003). Our data provides evidence for the possible involvement of P2X receptors in the generation of rabbit CB chemosensory activity, as in most of the species studied.

Both ATP (Buttigieg and Nurse, 2004) and adenosine (Conde and Monteiro, 2004) are released from CB during hypoxia. It has been suggested that adenosine may mediate some of the effects of exogenously applied ATP in the rat CB (McQueen and Ribeiro, 1981, 1983; Runold et al., 1990; Conde et al., 2006). However, our results indicate that rabbit PG neurons are largely insensitive to AMP, as the cat PG neurons (Alcayaga et al., 2000a). Thus, our data provides further evidence for a postsynaptic involvement of P2 receptors in the generation of chemosensory activity in the CB.

4.5. Source of ACh and ATP evoked activity

In our preparation the application of ACh and ATP to the PG increases the frequency of discharge in the carotid nerve through the activation of receptors that must be located in the perikaryon of the sensory neurons. It has been shown that α7 subunits of the nicotinic ACh receptor are present both in the cat PG perikarya and their terminals in the CB (Shirahata et al., 1998), and that identified carotid chemosensory PG neurons respond with depolarization and increased discharge to applications of ACh to their perikaryon (Varas et al., 2003). Similarly, nucleotide P2X receptor subunits have been demonstrated in the perikaryon of rat petrosal neurons and their terminals in the CB (Prasad et al., 2001), and identified cat chemosensory PG neurons are depolarized and discharge action potentials when ATP is applied to their perikaryon (Varas et al., 2003). Moreover, cat PG neurons in tissue culture respond to applications of ACh and ATP with inward currents that are completely blocked by suramin and hexamethonium, respectively (Alcayaga et al., 2007). Thus, PG neurons perikarya and their terminals appear to share similar properties with respect to the receptors expressed. To the best of our knowledge there is no available information on the presence or possible functional role of nucleotide or ACh receptors in the terminals of PG neurons in the nucleus of the solitary tract. Moreover, ACh or ATP had no effect on the discharge when the drugs were applied over the glossopharyngeal nerve or its branches and when the glossopharyngeal nerve was crushed at its apparent origin in the ganglion. Thus, the responses recorded in the carotid nerve in response to applications of ACh and ATP to the PG neurons must arise from the activation of receptors present on the perikaryon, and that those responses must resemble the effects of the transmitters in the peripheral terminals.

The PG provides sensory innervation to the carotid sinus and CB through the carotid nerve. Because our recordings were obtained from the whole population of axons of the carotid nerve, the activity evoked by ACh and ATP could be conveyed both by barosensory and chemosensory units. However, cat carotid barosensory activity is insensitive to ACh (McQueen, 1980), and intracellular recordings of identified cat carotid barosensory neurons in the PG show that they are unresponsive to ACh applied to the soma (Varas et al., 2003). On the other hand, most cat chemosensory neurons respond to ATP applications to the soma, and the vast majority of these cells also respond to ACh (Varas et al., 2003). Thus, although we cannot rule out the participation of barosensory neurons in the generation of the recorded activity, we suggest that the activation of chemosensory neurons in the PG is the main source of the activity elicited by ACh and ATP.

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