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Research Report

ATP- and ACh-induced responses in isolated cat petrosal ganglion neurons

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ABSTRACT

Chemoreceptor (glomus) cells of the carotid body are synaptically connected to the sensory nerve endings of petrosal ganglion (PG) neurons. In response to natural stimuli, the glomus cells release transmitters, which acting on the nerve terminals of petrosal neurons increases the chemosensory afferent discharge. Among several transmitter molecules present in glomus cells, acetylcholine (ACh) and adenosine 5′-triphosphate (ATP) are considered to act as excitatory transmitter in this synapse. To test if ACh and ATP play a role as excitatory transmitters in the cat CB, we recorded the electrophysiological responses from PG neurons cultured in vitro. Under voltage clamp, ATP induces a concentration-dependent inward current that partially desensitizes during 20–30 s application pulses. The ATP-induced current has a threshold near 100 nM and saturates between 20–50 μM. ACh induces a fast, inactivating inward current, with a threshold between 10–50 μM, and saturates around 1 mM. A large part of the population of PG neurons (60%) respond to both ATP and ACh. Present results support the hypothesis that ACh and ATP act as excitatory transmitters between cat glomus cells and PG neurons.

1. Introduction

Petrosal ganglion (PG) neurons innervate the carotid bifurcation through the carotid sinus nerve, and the pharynx and tongue through the glossopharyngeal branch of the glossopharyngeal nerve. The carotid sinus baroreceptors and pharyngeal mechanoreceptors are activated by the mechanical deformation of their peripheral endings (Kraske et al., 1998; McCarter et al., 1999). On the contrary, petrosal neurons projecting to the carotid body (CB) and the tongue are synaptically activated by one or more transmitters released by chemoreceptor cells (Gonzalez et al., 1994; Koga and Bradley, 2000). The identity of the transmitters involved in the activation of the sensory fibers innervating the CB is not completely known. Several molecules, including acetylcholine (ACh), dopamine, serotonin, γ-aminobutyric acid, substance P and adenosine 5′-triphosphate (ATP), had been suggested to participate in this synapse (Gonzalez et al., 1994). However, new evidence has suggested that both ACh and ATP mediate chemoreception in the cat and rat carotid body (Iturriaga and Alcayaga, 2004; Nurse, 2005). Indeed, the application of ACh to the isolated cat PG in vitro increases the frequency of neural

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Abbreviations: ACh, Acetylcholine; ATP, Adenosine 5′-triphosphate; CB, Carotid body; PG, Petrosal ganglion.

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discharge in the carotid sinus nerve but has minimal or no effect on the discharge of the glossopharyngeal branch (Alcayaga et al., 1998, 2000b). Moreover, ACh induces depolarization and evokes action potentials in cultured neurons from rat petrosal–jugular ganglion (Zhong and Nurse, 1997; Zhang et al., 2000) and cat PG neurons (Varas et al., 2000), although hyperpolarization, depolarization and biphasic responses had also been observed in cat PG neurons (Shirahata et al., 2000). In the cat PG in vitro, ATP increases the frequency of discharge in the carotid sinus nerve and, to a lesser extent in the glossopharyngeal branch (Alcayaga et al., 2000a), an effect that was not antagonized by Reactive Blue 2, a P2Y receptor blocker. Similarly, in isolated rat petrosal–jugular neurons, ATP depolarized and evoked action potentials, an effect blocked by the P2 receptor blocker suramin (Zhang et al., 2000). In co-cultures of rat petrosal–jugular neurons and CB cells, basal discharges and hypoxia-induced action potentials in the neurons are partially blocked by suramin or hexamethonium but completely inhibited by the simultaneous application of both blockers (Zhang et al., 2000). Recently, we found that hexamethonium and suramin reversibly and partially antagonized the increased discharges evoked by stop flow and acidosis in identified chemosensory neurons from the PG, while the simultaneous application of both drugs abolished the responses (Varas et al., 2003).

Most of the information regarding the electrophysiological effects of ATP and ACh on petrosal neurons has been obtained in cultured rat petrosal–jugular ganglion neurons. Indeed, Zhong and Nurse (1997) found that ACh induces a hexamethonium-sensitive inward current in rat petrosal–jugular ganglion neurons. However, depolarization and hyperpolarization have been reported in response to ACh in cat PG neurons (Shirahata et al., 2000), suggesting that species differences may account for different effects of some putative transmitters in the CB (see Iturriaga and Alcayaga, 2004). ATP induces a concentration-dependent, slowly inactivating, inward current in rat petrosal–jugular ganglion neurons (Zhang et al., 2000), but the effects of ATP on membrane currents of cat PG neurons have not been yet studied. Since several physiological and pharmacological experiments performed in cats had contributed to a deeper understanding of the CB chemosensory process, we decided to study the electrophysiological responses evoked by ACh and ATP in cultured cat PG neurons. In order to determine if isolated PG neurons may respond to the application of both ACh and ATP, we used the whole-cell configuration of the patch-clamp technique.

2. Results

PG neurons were isolated and maintained under culture conditions for 1–12 days. Medium-sized neurons (soma diameter: 30–70 μm) were recorded using the whole-cell configuration of the patch-clamp technique. Under current clamp conditions, most cells showed a resting membrane potential (V_m) near −60 mV, and under voltage clamp neurons showed zero net current at the same holding potential (V_H = −60 mV). Therefore, voltage clamp recordings were performed at V_H = −60 mV. No differences in resting membrane potential were observed between neurons from cultures of different ages.

2.1. *ATP-induced responses*

The continuous application of ATP through a pipette located close to the recorded neuron (~100 μm) for 10 to 30 s produced depolarization and/or inwardly directed current in 75.4% of the voltage-clamped cultured petrosal neurons studied (43 out of 57). Fig. 1 shows the changes in membrane potential (V_m) and whole-cell current (I_m) elicited by 2–20 μM ATP application in one neuron (soma diameter: 50 μm). ATP-induced depolarization developed with almost no delay and was maintained during the entire period of the ATP application (5–20 s). At concentrations of 5 μM ATP or higher, the membrane depolarization reached a threshold value (~50 mV) and bursts of action potentials were fired. It is noteworthy that 20 μM ATP depolarized the neurons to a level where action potential firing stopped and resumed only when the cell repolarized to a certain level (Fig. 1, upper panel). Similarly, during continuous ATP application inwardly directed currents were rapidly established and persisted during the application of the stimuli, presenting little or no desensitization (Figs. 1 and 2). Upon the conclusion of the ATP superfusion pulse, both V_m and I_m returned to the resting values with a time course that depended on the magnitude of the evoked response. Fig. 2 shows the inwardly directed currents induced by 30 s ATP pulses of increasing concentration (0.1–20 μM). The magnitude of the inward current elicited by ATP increased in a concentration-dependent manner (Figs. 1 and 2), showing a threshold between 0.1 and 0.2 μM, and subsided rapidly after the finalization of the pulse. In the recording showed in Fig. 2, corresponding to a neuron of 60 μm diameter, the ATP-induced current presented a low degree of desensitization, equivalent to about the 10–20% of the peak current for the largest ATP concentration (Fig. 2). Few neurons (3 out of 43) showed inwardly directed currents with a faster desensitization kinetics (not shown), suggesting heterogeneous purinergic receptor expression in at least some cat PG neurons.

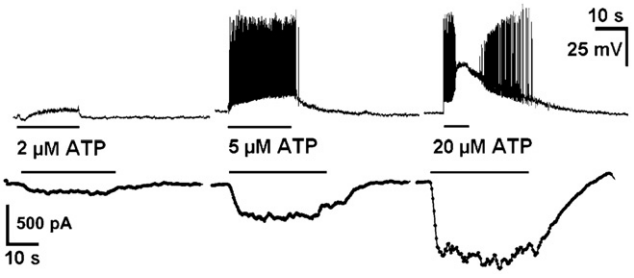

Fig. 1 – ATP-induced voltage (upper panel) and current (lower panel) responses recorded from the same cell in nominal Mg^{2+}-free external solution. Application of increasing ATP concentration induces depolarization and the discharge of action potentials, and a sustained inward current that persisted during the whole ATP application period (bar). Upper panel: resting membrane potential = −60 mV; lower panel: membrane holding potential = −60 mV.

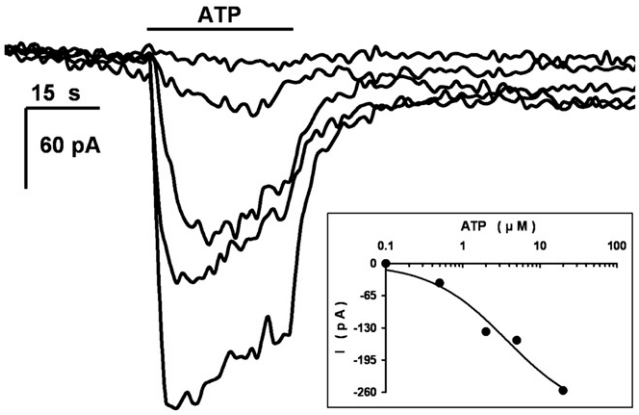

Fig. 2 – ATP-induced currents on a voltage-clamped petrosal ganglion neuron. ATP pulses (30 s) induced a sustained inward current, presenting a fast onset, little desensitization and a fast return after cessation of the stimulus. Currents were measured in nominal Mg^{2+}-free external solution. V_H = –60 mV. Inset shows the plot of induced peak currents as a function of ATP concentration. Data were fitted to the sigmoid equation $I = I_{max}/[1+\{EC_{50}/[ATP]^n\}]$. EC_{50}, median effective concentration. n, Hill coefficient.

Since the external concentration of Mg^{2+} modifies the sensitivity of P2X receptors to ATP in nodose ganglion neurons (Li et al., 1997), we determined the sensitivity to ATP in two different conditions: a 0.86-mM Mg^{2+} external solution, and in a nominal free-Mg^{2+} external solution. With 0.86 mM external Mg^{2+}, the ATP concentration–response curve (R^2 = 0.977) shows a Hill coefficient of 2.4±0.2 and an EC_{50} of 6.1±0.3 μM (n = 4), while in nominal free-Mg^{2+} external solution the curve (R^2 = 0.988) shows a Hill coefficient of 1.1±0.2 and an EC_{50} of 2.1±0.3 μM (n = 7, Fig. 3). As is shown in Fig. 3, the nominal absence of external Mg^{2+} shifted the concentration–response ATP curve to the left ($P < 0.05$; one-way ANOVA), indicating that cultured cat PG neurons have a higher sensitivity to external ATP in Mg^{2+}-free conditions.

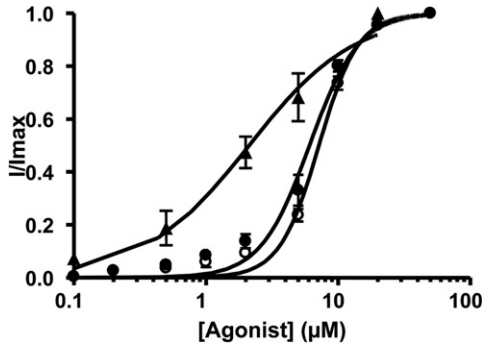

Fig. 3 – Mean concentration–response curves for currents evoked by ATP and α,β-methylene ATP. Normalized peak currents elicited by increasing concentrations of ATP in presence (Mg^{2+} = 0.86 mM, filled circles, n = 4) and absence of magnesium (triangles, n = 7) and α,β-methylene ATP (empty circles, n = 3; Mg^{2+} = 0.86 mM) are plotted. Data were fitted by the sigmoid equation $I/I_{max} = 1/[1+\{EC_{50}/[X]^n\}]$. $[X]$ = concentration of the agonist. EC_{50}, median effective concentration. n, Hill coefficient.

Whole-cell inward currents elicited by 20 μM ATP were measured under voltage clamp conditions at membrane holding potentials ranging from –100 to +40 mV. Fig. 4 shows the currents evoked by ATP in a single neuron and the mean current ($-I/I_{max}$) measured in 3 cells at each holding voltage normalized to the maximum peak current (I_{max}) measured at –100 mV. The macroscopic currents display a clear ohmic behavior for holding potentials between –100 and 0 mV, while at positive holding potentials the outwardly evoked currents were largely reduced, showing a significant inward rectification. The ATP voltage–current relationship showed a near zero (3.1±1.6 mV) reversal potential (Fig. 4, inset), suggesting that extracellular ATP induces an unspecific cationic current in cultured PG neurons.

After 5 min superfusion with the P2 receptor blocker suramin (20 μM), currents elicited by 10 μM ATP were reversibly abolished (Fig. 5A). To further characterize the subtype of P2X receptors mainly expressed in cat PGs, the effects of the P2X agonist α,β-methylene ATP were studied. Under voltage clamp, application of α,β-methylene ATP mimicked the effect of ATP, producing concentration-dependent increases of inwardly directed currents with fast onset and little or no desensitization, and a threshold for evoked currents near to 1 μM (Fig. 6). At the end of the α,β-methylene application, the membrane current returned to resting levels rapidly, showing a kinetics similar to those currents evoked by ATP (Fig. 6). A sigmoid saturating current–concentration relationship of a single neuron to increasing concentrations of α,β-methylene ATP (0.05 to 100 μM) is shown in the inset of Fig. 6. The current elicited by α,β-methylene ATP in 3 cells showed a threshold at 0.1 μM and reach an apparent

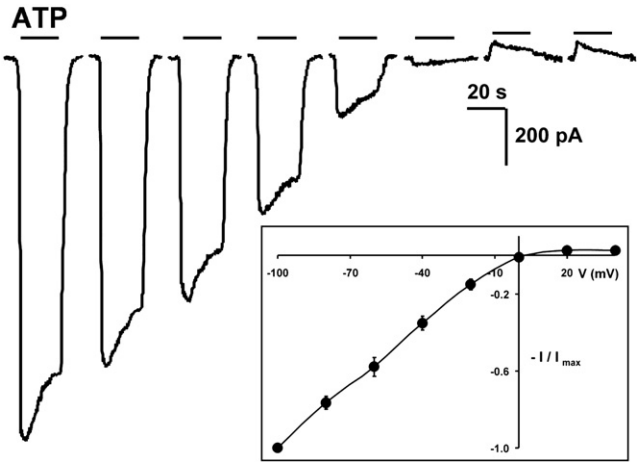

Fig. 4 – Current–voltage relationship of ATP-induced responses. Records of currents evoked by 20 μM ATP (bars) at membrane holding potentials ranging from –100 to 40 mV, in 20-mV steps. The inset graph shows the measured peak current (I) standardized to the peak current evoked at 101 mV (I_{max}) plotted as function of the holding membrane potential. ATP was added for 10 s and external solution contains 0.86 mM Mg^{2+}. Dots represent the mean current and the bars the standard error (n = 3).

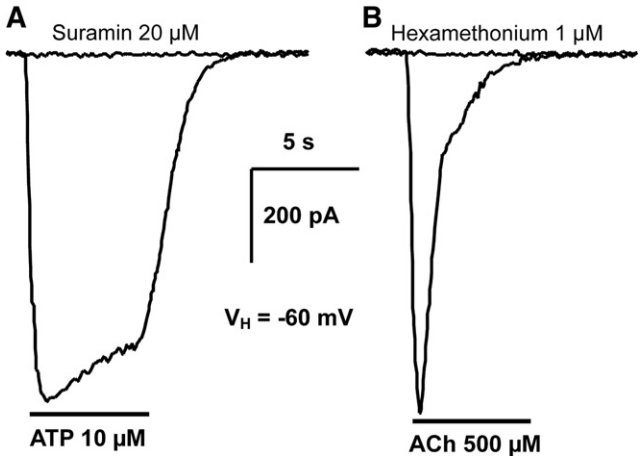

Fig. 5 – Antagonists suppress inwardly directed currents on PG neurons. (A) In the presence of 20 μM suramin, a P2X antagonist, a total inhibition of the ATP-induced current is observed. (B) Low concentration (1 μM) of hexamethonium added to the external solution completely abolished the inward current elicited by ACh. V_H = –60 mV. External solution contains 0.86 mM Mg^{2+}.

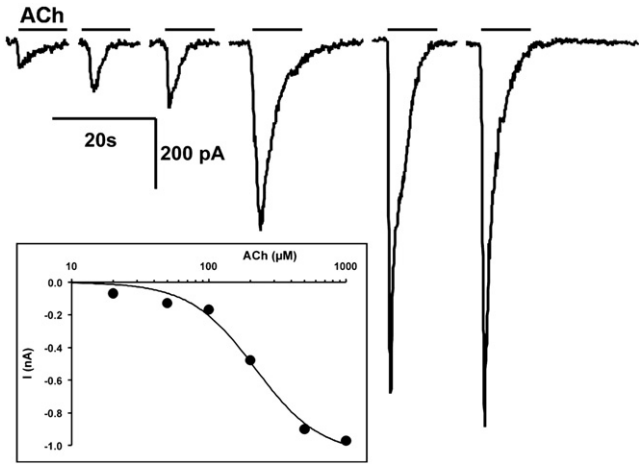

Fig. 7 – Inward currents induced by ACh at V_H = –60 mV. Currents of increasing amplitude obtained by the application of ACh for 10 s (bars). A fast temporal course for the onset and desensitization of the measured currents is observed. Whole-cell currents results on a saturating sigmoid curve ($I = I_{max}/[1 + \{EC_{50}/[ACh]^n\}]$) as the externally applied ACh concentration increases (inset). EC_{50}, median effective concentration. n, Hill coefficient. External solution contains 0.86 mM Mg^{2+}.

maximal effect at a concentration of 20 μM (Fig. 3). The mean concentration–response curve for α,β-methylene ATP in 0.86 mM external Mg^{2+} was fitted to a sigmoid curve (R^2 = 0.992) with a Hill coefficient of 2.9 ± 0.2 and an EC_{50} of 7.2 ± 0.2 μM (Fig. 3; n = 3). The analysis of ATP and α,β-methylene ATP concentration–response relationships in the presence of extracellular Mg^{2+} indicates that neither EC_{50}s nor Hill coefficients were statistically different ($P > 0.05$; ANOVA).

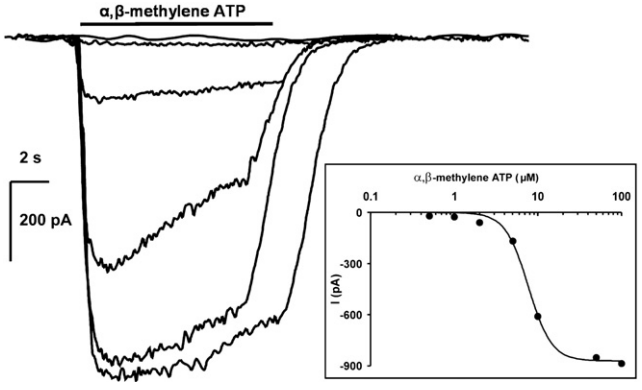

Fig. 6 – α,β-Methylene ATP elicits inward currents on a petrosal ganglion neuron at V_H = –60 mV. Current records measured for increasing concentrations of α,β-methylene ATP showing a fast onset, little desensitization and fast return to basal conditions after the end of the stimulus. Inset shows a sigmoid shape curve results when normalized currents are plotted as a function of the externally applied α,β-methylene ATP concentration ($I = I_{max}/[1 + \{EC_{50}/[\alpha,\beta\text{-methylene ATP}]^n\}]$). EC_{50}, median effective concentration. n, Hill coefficient. External Mg^{2+} concentration was 0.86 mM.

2.2. *Acetylcholine-induced currents*

In 39 out of 57 (68.4%) PG neurons voltage-clamped at –60 mV, ACh (20–1,000 μM) evoked concentration-dependent, fast desensitizing inward whole-cell currents (Figs. 5B and 7). The peak currents induced by increasing ACh concentrations (20–1,000 μM) showed a sigmoid concentration–response curve, with a threshold near 20 μM and a saturation value of about 500 μM (Fig. 7). The mean concentration–response curve elicited by several concentrations of ACh (R^2 = 0.972; n = 8) had an EC_{50} of 179 ± 8.4 μM and a Hill coefficient of 2.4 ± 0.3 (Fig. 8).

Fig. 9 shows the I–V curve for currents evoked by 1 mM ACh (10 s pulse) measured at membrane holding potentials ranging from –100 to +40 mV, in 20 mV steps. The peak currents (I)

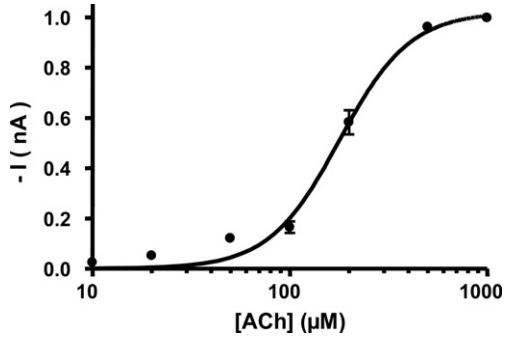

Fig. 8 – PG neurons concentration–response curve to external ACh. Peak inward currents as a function of external ACh concentration is plotted. EC_{50} and Hill coefficient are estimated from the sigmoid curve fitted by the equation $I = I_{max}/[1 + \{EC_{50}/[ACh]^n\}]$. Each point corresponds to average and standard error of eight experiments; V_H = –60 mV. EC_{50}, median effective concentration. n, Hill coefficient.

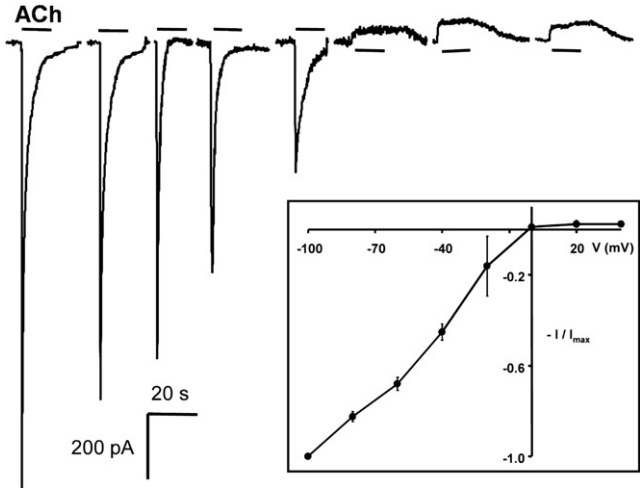

Fig. 9 – Current–voltage relationship in the presence of ACh. Whole-cell current evoked by 1 mM ACh pulses for 10 s (bar) measured at holding potentials from −100 to 40 mV (20 mV steps) are shown. In the inset, peak currents (*I*) standardized by the peak current evoked at $V_H = -100$ mV (I_{max}) are plotted as a function of the holding membrane potential. External solution contains 0.86 mM Mg^{2+}. Dots represent the mean current and the bars the standard error ($n=3$).

standardized to the maximum peak current (I_{max}) measured at −100 mV were recorded in 3 cells. The currents elicited by ACh were linearly related to the membrane potential from −100 to 0 mV range, whereas at positive potentials a strong inward rectification appeared (Fig. 9, inset). The reversal potential calculated from the *I–V* ACh curve was 0.1±1.2 mV, suggesting that ACh evoked an unspecific cationic current. The inward currents evoked by ACh were reversibly abolished when the nicotinic receptor blocker hexamethonium (1 μM) was added 3 min before to the external solution (Fig. 5B). Since we recently reported an electrophysiological characterization of the ACh-evoked currents using different nicotinic ACh receptor agonist and antagonist in the same preparation (Varas et al., 2006), no further attempts to describe the ACh-evoked currents were made.

2.3. *Acetylcholine and ATP-induced currents*

Interestingly, 34 out of 57 (59.6%) PG neurons were sensitive to both ATP and ACh, while 9 (15.8%) and 5 (8.8%) responded only to ATP or ACh, respectively. The remaining neurons studied (9 out of 57; 15.8%) were insensitive to both molecules.

3. Discussion

The main finding of the present study is that a substantial proportion of cultured isolated cat PG neurons (59.6%) respond to both ATP and ACh, the putative excitatory transmitters in CB neurotransmission (Zhang et al., 2000; Varas et al., 2003; Iturriaga and Alcayaga, 2004). In addition, some neurons were sensitive only to ATP (15.8%) or ACh (8.8%). Furthermore, present results show that responses to both ATP and ACh are mediated by inwardly directed ionic currents (therefore depolarizing and excitatory) through P2X purinergic and nicotinic cholinergic receptors, respectively.

Regarding to the ATP sensitivity, most of the PG neurons responded to ATP with fast sustained inward current, which amplitude increased in a concentration-dependent manner with an apparent threshold of 0.5 μM ATP and a maximum response attained at 10–50 μM ATP ($EC_{50}=6.1\pm0.3$ μM). The ATP-gated currents were mimicked by the P2X agonist α,β-methylene ATP and were reversibly inhibited by 20 μM suramin. Furthermore, the *I–V* curves for macroscopic currents showed strong inward rectification and a near zero reversal potential. Inward rectification has been reported for both, whole-cell currents through $P2X_2$ receptors (Evans et al., 1996; Zhou and Hume, 1998) and for single channel $P2X_4$ receptors (Negulyaev and Markwardt, 2000). Taking into account the kinetics of the ATP-induced current and the sensitivity to α,β-methylene ATP, our results suggest that heteromeric $P2X_{2,3}$ receptors are the main P2X receptor subtype in cat PG neurons (Fredholm et al., 1997; Lewis et al., 1995; North, 2002). Thus, present results agree with what was described for ATP sensitivity in isolated rat petrosal–jugular neurons (Zhang et al., 2000). In addition, immunoreactivity and presence of mRNA for the $P2X_2$ and $P2X_3$ proteins has been found in rat petrosal–jugular ganglia (Prasad et al., 2001; Zhang et al., 2000), as well as in other rat sensory ganglia (Dunn et al., 2001; Xiang et al., 1998).

We found that the number of ATP molecules needed to open the P2X channel is regulated by changes in external Mg^{2+} concentration (Fig. 6) as it has been found in rat nodose neurons (Li et al., 1997). Indeed, our results indicate that in 0.86 mM external Mg^{2+}, 2 ATP molecules are required for opening the P2X channel (Hill coefficient of 2.35), whereas only one molecule of ATP (Hill coefficient of 1.09) is needed in nominal-free external Mg^{2+}. These results suggest a change from a first order to a second order binding kinetics for ATP to the receptor in the absence and presence of Mg^{2+}, respectively. In addition, in Mg^{2+}-free conditions there is a left-shift of the concentration–response curve (EC_{50} of 2.1±0.3 μM vs. 6.1±0.3 μM, control and Mg^{2+}-free conditions, respectively), indicating that cultured cat PG neurons showed a higher sensitivity to external ATP concentration in the absence of external Mg^{2+}. A similar Mg^{2+}-mediated reduction of sensitivity to ATP has been described in rat nodose ganglion neurons (Li et al., 1997). Moreover, a Mg^{2+}-mediated blockade of the $P2X_4$ receptor currents (Negulyaev and Markwardt, 2000) and P2X currents in nodose ganglion neurons (Li et al., 1997), and an increased desensitization recovery time of $P2X_3$ receptors in rat dorsal root ganglia (Giniatullin et al., 2003) had been reported. However, because we did not record from the same neuron in normal (0.86 mM) an Mg^{2+}-free solutions, we cannot rule out a reduction in ATP-induced currents by Mg^{2+} in our preparation.

The presence of functional ATP purinoceptors in cat PG cultured neurons is consistent with our previous results showing that ATP applied to the isolated cat PG in vitro, evoked dose-dependent increases in the frequency of discharge in the carotid sinus nerve (Alcayaga et al., 2000a). Moreover, intracellular recordings from chemosensory identified petrosal neurons functionally connected to the CB in vitro

shows that application of ATP to the soma generates membrane depolarization and firing action potentials, responses that are inhibited by suramin (Varas et al., 2003).

On the other hand, present results also show that ACh evoked fast inactivated inward currents in ~65% of the petrosal neurons studied, in agreement with previous studies in cat PG (Varas et al., 2000, 2006) and rat (Zhong and Nurse, 1997) cultured petrosal–jugular neurons. The ACh-evoked current had a threshold of ~20 μM and reached saturation at 500 μM ACh (EC_{50}=196±34.2 μM). Therefore, the electrophysiological data on petrosal neurons obtained from adults cats (see also Varas et al., 2006) are in close agreement with those reported for petrosal–jugular neurons of perinatal rats (Zhong and Nurse, 1997; Koga and Bradley, 2000). In addition, our results showing that the ACh-evoked current is reversibly inhibited by the nicotinic receptor blocker hexamethonium (1 μM) agree with our previous studies performed in the entire cat PG (Alcayaga et al., 1998). Hexamethonium sensitivity of the ACh-evoked current has also been reported on rat jugular–petrosal neurons cultured alone (Zhong and Nurse, 1997) or in the presence of CB cells (Zhang et al., 2000). However, a much higher concentration of hexamethonium (200 μM) was required to block the responses in the rat petrosal–jugular neurons (Zhong and Nurse, 1997; Zhang et al., 2000). Additional evidence for the participation of nicotinic ACh receptors in the response to ACh-evoked currents is its reversal potential (near zero mV) and the strong inward rectification at positive potentials (Fig. 9), a functional characteristic of nicotinic ACh receptors that has been found in different preparations (Itier and Bertrand, 2001; McGehee and Role, 1995) including rat petrosal–jugular neurons in culture (Zhong and Nurse, 1997). Recently, we reported that the fast inactivated inward current evoked by ACh is mimicked by the nicotinic ACh receptor agonist cytisine and its bromo derivatives (Varas et al., 2006), which have been used to characterize subtypes of nicotinic ACh receptors (Houlihan et al., 2001; Slater et al., 2003). The currents elicited by ACh, cytisine and its bromo derivatives were reversibly abolished by 1 μM hexamethonium or 10 nM α-bungarotoxin (Varas et al., 2006). On the other hand, immunohistochemistry studies show the presence of the α7 subunit of the nicotinic ACh receptor in both nerve endings within the CB as well as in the soma of cat PG neurons (Shirahata et al., 1998). Accordingly, it is likely that homomeric α7 neuronal nicotinic ACh receptors predominate in cat PG neurons in culture. Nevertheless, since PG neurons express other neuronal nicotinic receptor subtypes such as α3 and α4, and β2 (Shirahata et al., 1998), it is possible that heteromeric receptors may also contribute to the whole current elicited by ACh.

In the present study, we found that roughly 60% of the cultured cat PG neurons respond to both ATP and ACh with fast inward currents and action potentials, mediated by the activation of P2X and nicotinic ACh receptors, respectively (Fig. 5). These results agree with our previous study on neurons from the cat PG functionally connected with the CB (Varas et al., 2003). In that preparation, 24 out of 27 identified carotid chemosensory PG neurons responded to both ATP and ACh increasing their action potential discharge frequency (Varas et al., 2003). Functional chemosensory synapses have been found in rat co-cultures of CB cells and petrosal–jugular neurons (Zhang et al., 2000), suggesting that cultured neurons may retain most of their properties described for intact preparations. Furthermore, in those functional chemosensory co-cultures, the response to hypoxia recorded in the neurons was blocked by the simultaneously application of P2X and nicotinic ACh receptor antagonist (Zhang et al., 2000). Thus, it is highly probable that at least some of the recorded ATP- and ACh-sensitive PG neurons in the present study correspond to chemosensory neurons that project to the CB. However, excitatory responses to ATP have been also found in barosensory PG neurons projecting to the carotid sinus (Varas et al., 2003) and in the cat glossopharyngeal branch fibers (Alcayaga et al., 2000a). In addition, even when we reported only vestigial ACh sensitivity in the glossopharyngeal branch (Alcayaga et al., 1998), the idea that ACh acts as an excitatory transmitter between gustatory chemoreceptor cells of the tongue and glossopharyngeal nerve endings was proposed decades ago (Landgren et al., 1954). Moreover, Fluorogold-labeled rat jugular–petrosal neurons that project to the tongue respond to ACh with inward currents (Koga and Bradley, 2000). Therefore, it is necessary to determine whether co-transmission of ATP and ACh may play a role in glossopharyngeal gustatory chemosensory transmission. In previous studies, we discussed the possibility that culture conditions could modify the survival and/or the functional phenotype of PG in culture (Varas et al., 2000, 2006). A preparation similar to that used by Belmonte and Gallego (1983) and Varas et al. (2003), which allowed single-cell recordings of PG neurons without disrupting the functional communication between PG neurons and their targets, could provide information about how widespread is this modality of fast-excitatory transmission. Is it a particularity of the CB synapse or common property of the PG neurons?

In summary, present results show that under voltage clamp, ATP induces concentration-dependent inward currents that partly desensitize during the ATP application, with a threshold near 100 nM, and saturation between 20 and 50 μM. The time course, sensitivity and magnitude of this response were largely mimicked by α,β-methylene ATP, suggesting the involvement of $P2X_{2,3}$ receptors in the generation of the response. ACh induces fast, inactivating inward currents, with a threshold at 10–50 μM, and saturation between 1 and 5 mM, an effect completely blocked by a nicotinic ACh receptor blocker. A large part of the neuronal population responded to both ATP and ACh (58.6%), while some neurons were only sensitive to ATP (15.8%) or ACh (8.8%); the rest (16.8%) corresponds to cells insensitive to ACh and ATP. Present results support the hypothesis that ACh and ATP may act as excitatory transmitters in the synapse between cat glomus cells and PG neurons.

4. Experimental procedures

4.1. *Cell culture*

Adult cats of either sex, weighing from 2 to 3 kg, were anesthetized with sodium pentobarbitone (40 mg/kg, i.p.). Additional doses (12 mg/kg, i.v.) were provided during surgery to maintain an adequate level of surgical anesthesia. At the

end of the surgical procedure, the animals were sacrificed with an intravenous anesthetic overdose. The experimental protocol was approved by the Ethical Committees of the Facultad de Ciencias of the Universidad de Chile and the Facultad de Ciencias Biológicas of the P. Universidad Católica de Chile and meets the guidelines of the National Fund for Scientific and Technological Research (FONDECYT), Chile. Petrosal ganglia (PG) were removed bilaterally as previously described (Varas et al., 2000) and kept in ice-chilled Ca^{2+}-, Mg^{2+}-free Hanks' solution (mHBSS), at pH 7.43. The PGs were first cleaned from surrounding connective tissue, minced into small pieces (15–20) with scissors and then enzymatically dissociated in mHBSS supplemented with collagenase (0.1%), trypsin (0.05%) and DNAse (150 U/ml). The dissociation, carried out under constant agitation at 38 °C for 30–60 min, was stopped by adding soybean trypsin inhibitor and fetal bovine serum (FBS) to attain final concentrations of 0.1 mg/ml and 10%, respectively. The cell suspension was centrifuged at 4,000×*g* for 10 min, and the pellet resuspended in Ham's F-12 supplemented with 20 mM HEPES, 10% heat inactivated horse serum, 10% FBS and 15 ng/ml of nerve growth factor (7S). Cells were plated on poly-L-lysine (0.1 mg/ml)-coated 35 mm Petri dishes and maintained in an incubator at 38 °C in water-saturated, 5% CO_2 in air atmosphere. After allowing cells to settle for three days, culture medium was changed every other day. Under these conditions, cells survived for several weeks.

4.2. *Electrophysiological recordings*

Culture medium was replaced by the external recording solution containing (in mM) 137 NaCl, 5.37 KCl, 1.26 $CaCl_2$, 0.81 Mg SO_4, 0.44 KH_2PO_4, 0.34 Na_2HPO_4, 4.17 $NaHCO_3$, 5 HEPES and 5.55 D-glucose, with pH adjusted to 7.43 at room temperature (20–25 °C). Alternatively, a nominal Mg^{2+}-free external solution was prepared (by adding no Mg^{2+} to the external solution) and used in some experiments. The dish was placed on the stage of an inverted microscope, and the solution was continuously changed with a two channel peristaltic pump (Masterflex C/L, Cole Parmer), at a rate of 1.2–1.5 ml/min. Patch-clamp borosilicate glass pipettes (GC150F-10, Warner Instruments Corp.), pulled and polished in a Flaming/Brown P-87 Micropipette Puller (Sutter Instrument Co.), were filled with the internal solution containing (in mM) 5 NaCl, 135 KCl, 1 $CaCl_2$, 11 EGTA, 10 HEPES, pH 7.2. Electrode resistance ranged from 1 to 5 MΩ. Using a multichannel perfusion system, testing solutions containing adenosine 5′-triphosphate (ATP; 0.1–100 μM), α,β-methylene ATP (0.1–100 μM) or acetylcholine (ACh; 0.2–1,000 μM) were applied in the surroundings of the recorded cell.

Under phase contrast microscopy, mainly round- to ovoid-shaped neurons (30 to 70 μm in major diameter) were chosen for tight GΩ seals. Seal formation and membrane breakthrough were monitored by observing under current-clamp mode the response to a 2- to 10-pA hyperpolarizing current. Whole-cell voltage or current clamp protocols were controlled online by an IBM-compatible PC. Voltage or current signals were amplified (PC 501A, Warner Instruments, USA), filtered at 5 kHz, digitized (LabMaster DMA, Scientific Solutions, USA) and stored in the computer or, alternatively, recorded on compact discs after digital encoding (Biologic DRA-400, France) for posterior analysis with BIO-TOOLS V2.06 program (Biologic, France). Cellular integrity and clamp parameters were frequently tested by the presence of properly clamped voltage-gated Na^+ and K^+ currents. Cells displaying abnormal and/or not in control currents were discharged. Voltage–current (*I–V*) curves for ATP or ACh were constructed by imposing membrane potentials ranging from −100 to +40 mV (in 20 mV steps magnitude) in the presence of external 1 μM TTX and 5 mM TEA. All experiments were performed at 20–25 °C.

4.3. *Statistical analysis*

The peak (*I*) currents elicited by several concentrations of ATP, α,β-methylene ATP and ACh were standardized to the maximal current (I_{max}) evoked by largest concentrations of these drugs. The peak standardized currents were fitted to the equation $I/I_{max}=1/[1+\{EC_{50}/[X]^n\}]$, and the median effective concentration (EC_{50}) and the Hill coefficient (*n*) were estimated. Differences between currents evoked under different experimental conditions were compared using one-way analysis of variance (ANOVA). All curve fitting and statistical calculations were performed with GraphPad Prism (GraphPad Software, USA).

4.4. *Drugs*

ACh, ATP and α,β-methylene ATP were freshly prepared in Hanks' solution, diluted to desired concentration (0.0001–1 mM) and delivered by gravity ejection from a nearby pipette positioned at approximately 100 μm from the cell surface. In order to block the responses evoked by ACh and ATP, we used the nicotinic receptor blocker hexamethonium and the P2 purinoceptor antagonist suramin. Both agents were diluted to required concentration in external (Hanks') solution and applied continuously through the conventional superfusion system. All chemicals were obtained from Sigma Chemical Co. (USA), except for the fetal bovine serum that was purchased from HyClone Laboratories (USA).

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REFERENCES

Alcayaga, J., Iturriaga, R., Varas, R., Arroyo, J., Zapata, P., 1998. Selective activation of carotid nerve fibers by acetylcholine applied to the cat petrosal ganglion in vitro. Brain Res. 786, 47–54.

Alcayaga, J., Cerpa, V., Retamal, M., Arroyo, J., Iturriaga, R., Zapata, P., 2000a. Adenosine triphosphate-induced peripheral nerve discharges generated from the cat petrosal ganglion in vitro. Neurosci. Lett. 282, 185–188.

Alcayaga, J., Varas, R., Arroyo, J., Iturriaga, R., Zapata, P., 2000b. Responses of petrosal ganglion neurons in vitro to hypoxic stimuli and putative transmitters. Adv. Exp. Med. Biol. 475, 389–396.

Belmonte, C., Gallego, R., 1983. Membrane properties of cat sensory neurones with chemoreceptor and baroreceptor endings. J. Physiol. 342, 603–614.

Dunn, P.M., Zhong, Y., Burnstock, G., 2001. P2X receptors in peripheral neurons. Prog. Neurobiol. 65, 107–134.

Evans, R.J., Lewis, C., Virginio, C., Lundstrom, K., Buell, G., Surprenant, A., North, R.A., 1996. Ionic permeability of, and divalent cation effects on, two ATP-gated cation channels (P2X receptors) expressed in mammalian cells. J. Physiol. 497, 413–422.

Fredholm, B.B., Abbracchio, M.P., Burnstock, G., Dubyak, G.R., Harden, T.K., Jacobson, K.A., Schwabe, U., Williams, M., 1997. Towards a revised nomenclature for P1 and P2 receptors. Trends Pharmacol. Sci. 18, 79–82.

Giniatullin, R., Sokolova, E., Nistri, A., 2003. Modulation of P2X3 receptors by Mg^{2+} on rat DRG neurons in culture. Neuropharmacology 44, 132–140.

Gonzalez, C., Almaraz, L., Obeso, A., Rigual, R., 1994. Carotid body chemoreceptors: from natural stimuli to sensory discharges. Physiol. Rev. 74, 829–898.

Houlihan, L.M., Slater, Y., Guerra, D.L., Peng, J.-H., Kuo, Y.-P., Lukas, R.J., Cassels, B.K., Bermúdez, I., 2001. Activity of cytisine and its brominated isosteres on recombinant human $\alpha7$, $\alpha4\beta2$ and $\alpha4\beta4$ nicotinic acetylcholine receptors. J. Neurochem. 78, 1029–1043.

Itier, V., Bertrand, D., 2001. Neuronal nicotinic receptors: from protein structure to function. FEBS Lett. 504, 118–125.

Iturriaga, R., Alcayaga, J., 2004. Neurotransmission in the carotid body: transmitters and modulators between glomus cells and petrosal ganglion nerve terminals. Brain Res. Rev. 47, 46–53.

Koga, T., Bradley, R.M., 2000. Biophysical properties and responses to transmitters of petrosal and geniculate ganglion neurons innervating the tongue. J. Neurophysiol. 84, 1404–1413.

Kraske, S., Cunningham, J.T., Hajduczok, G., Chapleau, M.W., Abboud, F.M., Wachtel, R.E., 1998. Mechanosensitive ion channels in putative aortic baroreceptor neurons. Am. J. Physiol. 275, H1497–H1501.

Landgren, S., Liljestrand, G., Zotterman, Y., 1954. Chemical transmission in taste fibre endings. Acta Physiol. Scand. 30, 105–114.

Lewis, C., Neidhart, S., Holy, C., North, R.A., Buell, G., Surprenant, A., 1995. Coexpression of P2X2 and P2X3 subunits can account for ATP-gated currents in sensory neurons. Nature 377, 432–435.

Li, C., Peoples, R.W., Weight, F.F., 1997. Mg^{2+} inhibition of ATP-activated current in rat nodose ganglion neurons: evidence that Mg^{2+} decreases the agonist affinity of the receptor. J. Neurophysiol. 77, 3391–3395.

McCarter, G.C., Reichling, D.B., Levine, J.D., 1999. Mechanical transduction by the rat dorsal root ganglion neurons in vitro. Neurosci. Lett. 273, 179–182.

McGehee, D.S., Role, L.W., 1995. Physiological diversity of nicotinic acetylcholine receptors expressed by vertebrate neurons. Annu. Rev. Physiol. 57, 521–546.

Negulyaev, Y.A., Markwardt, F., 2000. Block by extracellular Mg^{2+} of single human purinergic P2X4 receptor channels expressed in human embryonic kidney cells. Neurosci. Lett. 279, 165–168.

North, R.A., 2002. Molecular physiology of P2X receptors. Physiol. Rev. 82, 1013–1067.

Nurse, C.A., 2005. Neurotransmission and neuromodulation in the chemosensory carotid body. Auton. Neurosci. 120, 1–9.

Prasad, M., Fearon, I.M., Zhang, M., Laing, M., Vollmer, C., Nurse, C.A., 2001. Expression of P2X2 and P2X3 receptor subunits in rat carotid body afferent neurones: role of chemosensory signaling. J. Physiol. 537, 667–677.

Shirahata, M., Ishizawa, Y., Rudisill, M., Schofield, B., Fitzgerald, R.S., 1998. Presence of nicotinic acetylcholine receptors in cat carotid body afferent system. Brain Res. 814, 213–217.

Shirahata, M., Ishizawa, Y., Rudisill, M., Sham, J.S., Schofield, B., Fitzgerald, R.S., 2000. Acetylcholine sensitivity of cat petrosal ganglion neurons. Adv. Exp. Med. Biol. 475, 377–387.

Slater, Y., Houlihan, L.M., Maskell, P.D., Exley, R., Bermúdez, I., Lukas, R.J., Valdivia, A.C., Cassels, B.K., 2003. Halogenated cytisine derivatives as agonist at human neuronal nicotinic acetylcholine receptor subtypes. Neuropharmacology 44, 503–515.

Varas, R., Alcayaga, J., Zapata, P., 2000. Acetylcholine sensitivity in sensory neurons dissociated from the cat petrosal ganglion. Brain Res. 882, 201–205.

Varas, R., Alcayaga, J., Iturriaga, R., 2003. ACh and ATP mediate excitatory transmission in cat carotid identified chemoreceptor units in vitro. Brain Res. 988, 154–163.

Varas, R., Valdés, V., Iturriaga-Vásquez, P., Cassels, B.K., Iturriaga, R., Alcayaga, J., 2006. Electrophysiological characterization of nicotinic acetylcholine receptors in cat petrosal ganglion neurons in culture: effects of cytisine and its bromo derivatives. Brain Res. 1072, 72–78.

Xiang, Z., Bo, X., Burnstock, G., 1998. Localization of ATP-gated P2X receptor immunoreactivity in rat sensory and sympathetic ganglia. Neurosci. Lett. 256, 105–108.

Zhang, M., Zhong, H., Vollmer, C., Nurse, C.A., 2000. Co-release of ATP and ACh mediates hypoxic signalling at rat carotid body chemoreceptors. J. Physiol. 525, 143–158.

Zhong, H., Nurse, C.A., 1997. Nicotinic acetylcholine sensitivity of rat petrosal sensory neurons in dissociated cell culture. Brain Res. 766, 153–161.

Zhou, Z., Hume, R.I., 1998. Two mechanisms for inward rectification of current flow through the purinoceptor P2X2 class of ATP-gated channels. J. Physiol. 507, 353–364.