Electrophysiological characterization of nicotinic acetylcholine receptors in cat petrosal ganglion neurons in culture: Effects of cytisine and its bromo derivatives

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A R T I C L E I N F O

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Abbreviations:
3-Br-cy, 3-bromocytisine
5-Br-cy, 5-bromocytisine
ACh, acetylcholine
nAChR, nicotinic acetylcholine receptor
PG, petrosal ganglion

A B S T R A C T

Petrosal ganglion neurons are depolarized and fire action potentials in response to acetylcholine and nicotine. However, little is known about the subtype(s) of nicotinic acetylcholine receptors involved, although α4 and α7 subunits have been identified in petrosal ganglion neurons. Cytisine, an alkaloid unrelated to nicotine, and its bromo derivatives are agonists exhibiting different affinities, potencies and efficacies at nicotinic acetylcholine receptors containing α4 or α7 subunits. To characterize the receptors involved, we studied the effects of these agonists and the nicotinic acetylcholine receptor antagonists hexamethonium and α-bungarotoxin in isolated petrosal ganglion neurons. Petrosal ganglia were excised from anesthetized cats and cultured for up to 16 days. Using patch-clamp technique, we recorded whole-cell currents evoked by 5–10 s applications of acetylcholine, cytisine or its bromo derivatives. Agonists and antagonists were applied by gravity from a pipette near the neuron surface. Neurons responded to acetylcholine, cytisine, 3-bromocytisine and 5-bromocytisine with fast inward currents that desensitized during application of the stimuli and were reversibly blocked by 1 μM hexamethonium or 10 nM α-bungarotoxin. The order of potency of the agonists was 3-bromocytisine ≫ acetylcholine ≅ cytisine ≫ 5-bromocytisine, suggesting that homomeric α7 neuronal nicotinic receptors predominate in cat petrosal ganglion neurons in culture.

1. Introduction

The petrosal ganglion (PG) is the main sensory ganglion containing the perikarya of primary afferent neurons of the glossopharyngeal nerve, whose peripheral axons project either through the carotid sinus nerve or the glossopharyngeal branch (Alcayaga et al., 1996; Stensaas and Fidone, 1977). The carotid sinus nerve conveys chemo- and mechanosensory fibers from the carotid body and carotid sinus, respectively, while the glossopharyngeal branch contains chemo- and mechanosensory fibers from the tongue and the pharynx. The transduction of chemical

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stimuli involves specialized receptor cells in both the carotid body and the gustatory papillae.

The current model of chemotransduction in the carotid body states that chemoreceptor (glomus) cells release one or more transmitters, which in turn increase the frequency of sensory discharges originated from PG neurons peripheral endings (Iturriaga and Alcayaga, 2004). In response to hypoxia and hypercapnia, the carotid body releases acetylcholine (ACh) from chemoreceptor cells (Fitzgerald et al., 1999), and several studies indicate that ACh modulates the release of catecholamines from cat and rabbit carotid body chemoreceptor cells (Hirasawa et al., 2002; Ishizawa et al., 1996; Iturriaga et al., 2000; Shirahata et al., 1998).

Applications of ACh to the isolated rat and cat PG neurons in culture evoke inward currents, depolarization and action potentials, effects mimicked by nicotine and blocked by hexamethonium (Koga and Bradley, 2000; Varas et al., 2000; Zhong and Nurse, 1997). Moreover, applications of ACh to the superfused cat PG generate bursts of action potentials conducted along the carotid sinus nerve, while only a few spikes are observed in the glossopharyngeal branch in response to the highest doses of ACh (Alcayaga et al., 1998). The neural response evoked by ACh is dose-dependent and reversibly antagonized by hexamethonium and mecamylamine. Similarly, recordings from cat PG neurons functionally connected to the carotid body in vitro show that application of ACh to their somata depolarizes these neurons, having no effect on PG mechanosensory neurons (Varas et al., 2003). Thus, the electrophysiological data indicate that a population of PG neurons projecting through the carotid sinus nerve are selectively activated by ACh acting on nicotinic ACh receptors located in the somata of these neurons.

Nicotinic cholinergic receptors (nAChRs) are widely distributed in vertebrates, including different regions of the central and peripheral nervous systems (Hogg et al., 2003). Neuronal nAChRs are pentameric structures resulting from the ensemble of 12 subunits (α2–α10 and β2–β4), of which at least homomeric α7 and α9 and heteromeric $\alpha4\beta2$ and $\alpha2\beta4$ receptor subtypes are known to be present in other sensory ganglia (Genzen et al., 2001; Lips et al., 2002; Liu et al., 1998). The subunit composition of nACh heteromeric receptors determines features such as conductance and kinetics of desensitization (Fenster et al., 1997), Ca^{2+} permeability and agonist and/or antagonist affinities. Homomeric α7-nACh receptors have the highest Ca^{2+} permeability, the fastest desensitization kinetics of nACh receptors and are blocked by α-bungarotoxin. In contrast, heteromeric receptors have much lower Ca^{2+} permeability and are mostly insensitive to α-bungarotoxin (Fenster et al., 1997; Gotti et al., 1997). Immunohistochemical studies have shown the presence of α7 and α4 nACh subunits in the nerve endings as well as in the perikarya of cat PG neurons that innervate the carotid body, suggesting that α7 and $\alpha4\beta2$ receptors predominate in these neurons (Ishizawa et al., 1996; Shirahata et al., 1998). However, little is known about the functional type of nAChRs that mediate the electrophysiological response of PG neurons to ACh. Accordingly, we characterized the currents elicited by ACh in PG neurons using the nAChR agonist cytisine and its bromo derivatives 3-bromocytisine (3-Br-cy) and 5-bromocytisine (5-Br-cy) to distinguish between the α7 and $\alpha4\beta2$ nicotinic cholinergic receptor subtypes (Houlihan et al., 2001; Slater et al., 2003).

2. Results

2.1. ACh-evoked currents in cultured PG neurons

At an imposed membrane resting potential of −60 mV, ACh (0.02–1 mM) evoked fast-desensitizing, dose-dependent transient inward currents in 17 out of 24 (71%) PG neurons in culture (Figs. 1 and 3A). To avoid the effects of receptor desensitization, at least 5 min were allowed between successive ACh applications. The nicotinic blocker hexamethonium (1 μM) reversibly suppressed ACh-evoked inward currents in all the cases studied (Figs. 1A, B). Fig. 1 shows the inward currents induced by ACh in 3 different neurons. Superfusion for 3 min with Hanks' solution containing 1 μM hexamethonium completely abolished the inward current (Figs. 1A, B), an effect that was reversible in all the 17 neurons studied. Similarly, the nicotinic blocker α-bungarotoxin (10 nM) reversibly blocked the ACh-evoked inward current in 5 of 6 PG neurons studied (83%). Fig. 1C shows that the inward current evoked by ACh (500 μM) in one neuron was reversibly blocked by superfusion of α-bungarotoxin (10 nM) for 3 min. The blocking effect α-bungarotoxin was reversible in two neurons in which the recording was stable after a 10-min period of wash out.

2.2. Currents evoked by cytisine and its bromo derivatives

Cytisine and 3-Br-cy evoked dose-dependent, transient inward currents in 15 of the 17 (88%) neurons responding to ACh (Figs. 3B–D). In contrast, 5-Br-cy only induced currents in 10 out of the 15 (59%) neurons responding to ACh, all of which had responded to cytisine. It is worth mentioning that only those neurons that responded to ACh showed currents evoked by cytisine or its brominated derivatives. Fig. 2 shows a comparison of the responses evoked by 100 μM applications of ACh, cytisine, 5-Br-cy and 3-Br-cy in a single PG neuron. All these agents produced transient inward currents that receded within 4 s even when the agonists were still being applied to the neuron. For this single concentration of 100 μM, ACh and cytisine evoked currents of similar amplitude, while 3-Br-cy produced a larger inward current (about 140%) and 5-Br-cy evoked a smaller inward current (only 40%). Superfusion of 1 μM hexamethonium for 3 min completely and reversibly blocked the currents evoked by ACh, cytisine and 5-Br-cy, but failed to completely block the currents evoked by 3-Br-cy at concentrations higher than 100 μM (data not shown).

2.3. Dose–response curves evoked by ACh, cytisine and its bromo derivatives

Responses induced by increasing concentrations of ACh, cytisine, 3-Br-cy and 5-Br-cy are shown in Fig. 3. ACh and cytisine (20 μM–1 mM) produced dose-dependent inward currents of similar amplitude, with a threshold concentration of 10–20 μM and reaching maxima at concentrations

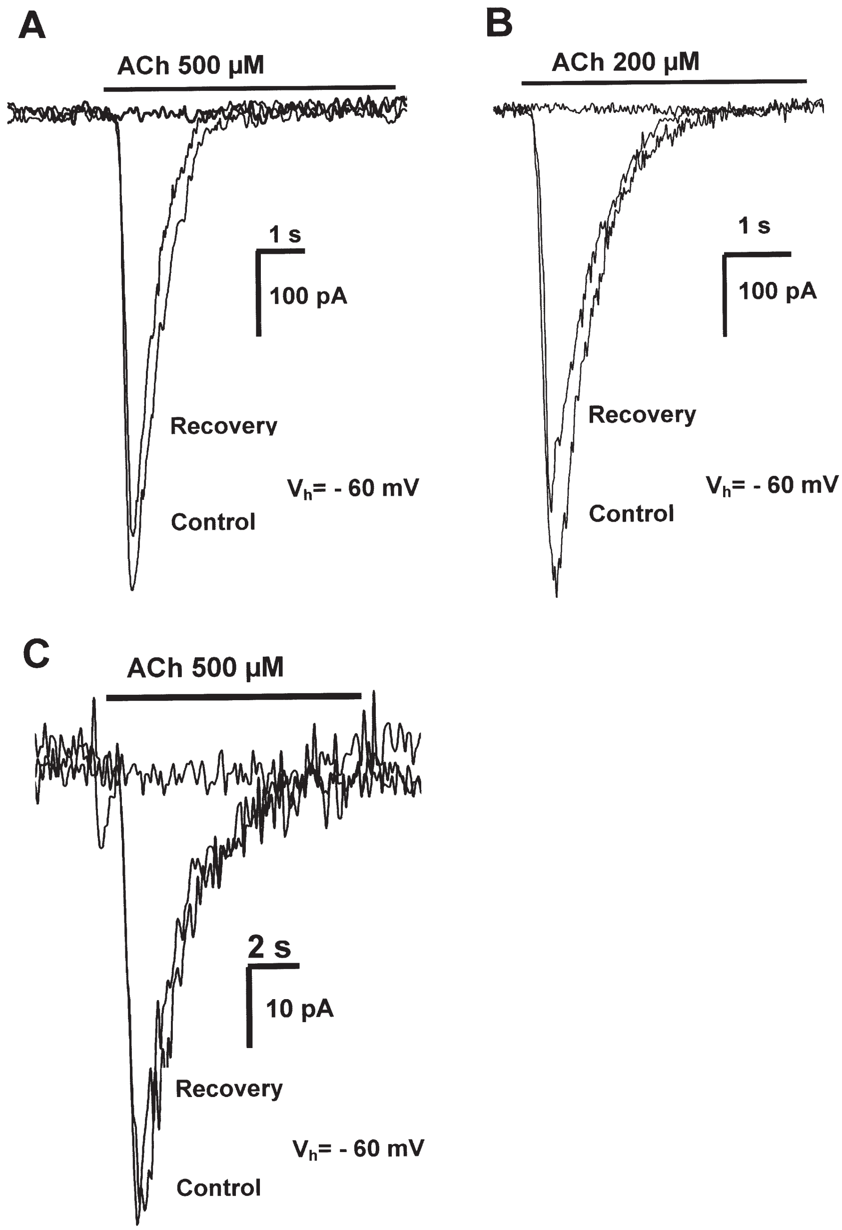

Fig. 1 – Inward currents evoked by ACh in three cultured cat PG neurons. Superfusion with Hanks' solution containing 200 or 500 μM ACh (pipette concentration) generates a rapidly inactivating inward current which is reversibly blocked by 1 μM hexamethonium (A, B) and 10 nM α-bungarotoxin (C). V_h, holding membrane potential at −60 mV.

near 0.5–1 mM (Figs. 3A, B). The maximal currents evoked by the highest concentrations of ACh and cytisine were in the order of 1 nA for neurons of about 45 μm of diameter. However, the neurons were more sensitive to 3-Br-cy, which produced currents of larger amplitude, reaching their maximum near 50–100 μM (Fig. 3C). In contrast, 5-Br-cy only induced very small inward currents in a neuron of similar size (Fig. 3D).

Fig. 4 summarizes the dose-dependent curves for inward currents evoked by ACh, cytisine, 3-Br-cy and 5-Br-cy in 6 neurons. The dose–response curves induced by ACh and cytisine had similar I_{max} (−1.02 vs. −1.12 nA) and ED_{50} (182 vs. 168 μM) (Fig. 4, Table 1), but that evoked by 3-Br-cy was shifted to the left by one order of magnitude (EC_{50} = 16.5 μM) and showed a larger I_{max} (−1.28 nA). In contrast, the currents evoked by 5-Br-cy attained a very small amplitude and showed a much higher EC_{50} (1264 μM) (Fig. 4, Table 1). Therefore, the rank order of potencies was: 3-Br-cy ≫ ACh ≅ cytisine ≫ 5-Br-cy.

3. Discussion

The present results show that ACh evoked transient inward currents in 71% of PG neurons studied. The proportion of ACh-sensitive neurons found here agrees with previous studies in rat (Zhong and Nurse, 1997) and cat cultured PG neurons

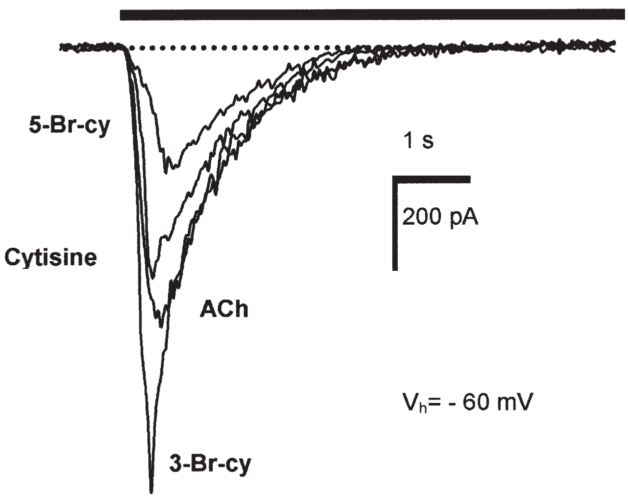

Fig. 2 – Inward currents elicited by 100 μM (in the pipette) concentrations of ACh, cytisine, 3-bromocytisine (3-Br-Cy) and 5-bromocytisine (5-Br-Cy) in a PG neuron in culture. The bar indicates the duration of the superfusion of the drugs. V_h, holding membrane potential at –60 mV.

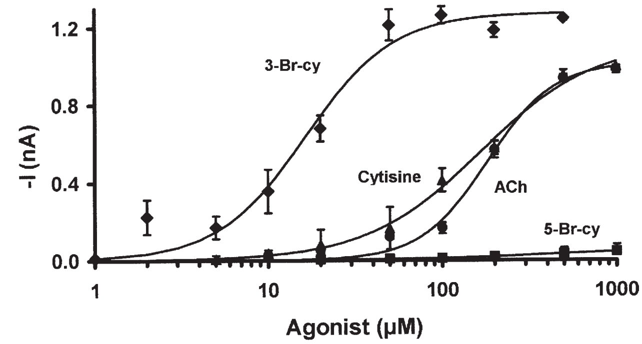

Fig. 4 – Dose–response curves for ACh (filled circles), cytisine (filled triangles), 3-bromocytisine (3-Br-cy, filled diamonds) and 5-bromocytisine (5-Br-cy, filled squares) in 6 PG neurons, at a holding membrane potential of –60 mV. The abscissa corresponds to the pipette concentration of the agonists.

(Varas et al., 2000). With a holding membrane potential of –60 mV, ACh evoked a fast inactivated inward current, whose amplitude showed a dose-dependent increase, with a

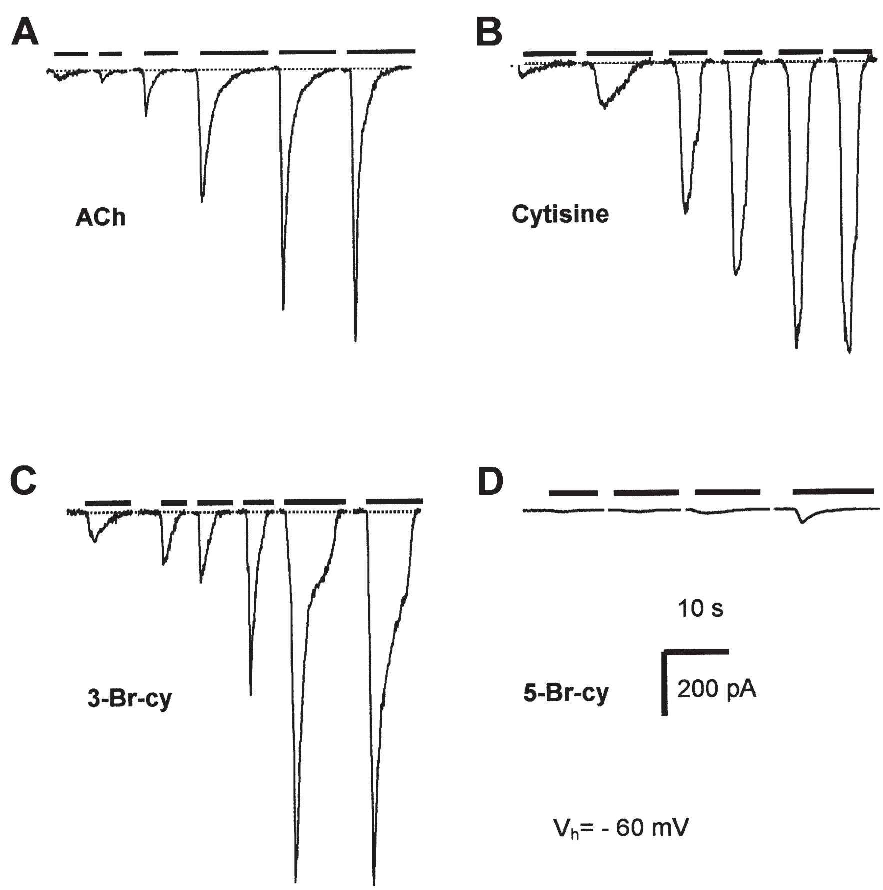

Fig. 3 – Whole cell inward currents evoked by increasing concentrations of ACh (A: 20, 50,100, 200, 500 and 1000 μM), cytisine (B: 10, 20, 100, 200, 500 and 1000 μM), 3-bromocytisine (C: 2, 5, 10, 20, 50 and 100 μM) and 5-bromocytisine (D: 100, 200, 500 and 1000 μM) in four cultured PG neurons of similar size (diameter 45 μm). Bars indicate the applications of the agonists. Successive doses were applied at 5 min intervals, at a holding membrane potential (V_h) of –60 mV.

Table 1 – Functional potency of ACh, cytisine and its isosters 3-bromocytisine (3-Br-cy) and 5-bromocytisine (5-Br-cy) on cultured cat PG neurons

	I_{max} (nA)	EC_{50} (μM)	n
ACh	–1.02	182.30	2.25
Cytisine	–1.12	167.65	1.32
3-Br-cy	–1.28	16.48	1.70
5-Br-cy	–0.10	1264.70	0.81

Estimated values obtained by fitting the dose–response curves to a non-linear regression according to the equation $I = I_{max} / [1 + (EC_{50} / X)^n]$. I_{max} = maximal current evoked by any given agonist. EC_{50} = concentration required to evoke half-maximal current amplitude. X = dose of a given agonist. n = calculated Hill coefficient (N = 6 neurons).

threshold of 10–20 μM, reaching a maximum between 0.5 and 1 mM. Thus, our data on PG neurons obtained from adult cats agree with those obtained from PG neurons of perinatal rats (Koga and Bradley, 2000; Zhong and Nurse, 1997). The ACh-induced current was reversibly blocked by 1 μM hexamethonium, a much lower concentration than the 200 μM required to block the ACh-induced inward current in rat PG neurons (Zhong and Nurse, 1997). Even more, 10 nM α-bungarotoxin blocked the current induced by ACh in 83% of the PG neurons studied.

The proportion of PG neurons projecting through the carotid sinus nerve represents approximately 15–20% of the total number of neurons in the ganglion, and only a half of the above proportion probably projects to the carotid body (Alcayaga et al., 1996; Berger, 1980; Claps and Torrealba, 1988; McDonald, 1983). However, more than two thirds of our neurons in culture showed sensitivity to ACh. A possibility is that PG neurons that project through the glossopharyngeal nerve also present ACh sensitivity. This agrees with the idea that ACh is the excitatory transmitter between the gustatory cells of the tongue and glossopharyngeal nerve endings (Landgren et al., 1954), and the observation that, when previously marked with Fluorogold applied to the tongue, 50% of the rat PG neurons in culture respond to ACh with inward currents (Koga and Bradley, 2000), similarly to the results reported here. Nevertheless, it is also possible that culture conditions favor the survival of PG neurons that project to the carotid body or the presence of trophic factors, as NGF, may induce the upregulation of nAChRs. In addition, modification of the trophic interaction between PG neurons and their target tissues due to culture conditions may induce an over-expression of nAChRs in the membranes of the PG neurons in culture. This idea is supported by the observation that scarce antidromic discharges are recorded from the glossopharyngeal branch in response to ACh applied to the PG superfused in vitro (Alcayaga et al., 1998).

A large percentage (88%) of the PG neurons that responded to ACh also responded to cytisine with inward currents of similar intensity and profile than those elicited by ACh. It must be noted that a long series of reports by Russian researchers led Anichkov and Belen'kii (1963) to conclude that cytisine is “one of the most powerful carotid chemoreceptor stimulants” and to use cytisine as a respiratory analeptic. Furthermore, cytisine applied to the superfused PG in vitro elicits antidromic discharges in the carotid sinus nerve (Alcayaga et al., unpublished observations).

Cytisine has a low micromolar affinity for α7 homomeric nAChRs (Flores et al., 1992; Pabreza et al., 1991), but it is a full agonist, with higher efficacy than ACh at these receptors. Otherwise, cytisine nanomolar affinity for α4β2 receptors is associated with a very low efficacy (0.04%) relative to that of ACh (Slater et al., 2003). The bromo isosteres of cytisine have shown widely different affinities and agonist potencies in recombinant human α7, α4β2 and α4β4 nAChRs expressed in cultured cells and in *Xenopus* oocytes (Houlihan et al., 2001; Slater et al., 2003). According to Houlihan et al. (2001) and Slater et al. (2003), cytisine and 3-Br-cy are full agonists at α7 nAChRs but partial agonists at α4β2 nAChRs. They also show high potency and efficacy at α4β4 nAChRs. 5-Bromocytisine has lower potency and is a partial agonist at α7 and α4β4 nAChRs, but it did not elicit any response at α4β2 nAChRs. Thus, cytisine and its derivatives are useful tools to distinguish between the α7 and α4β2 nicotinic cholinergic receptor subtypes.

Cytisine and its bromo derivatives evoked inward currents only in neurons activated by ACh, and these currents were blocked by hexamethonium. Cytisine shows high agonist potency ($EC_{50} \approx$ nM) at α4 and β2 subunit-containing nicotinic ACh receptors and much less potency ($EC_{50} \approx$ μM) at homomeric α7 nAChRs (Holladay et al., 1997; Houlihan et al., 2001; Slater et al., 2003). Interestingly, we observed that 3-Br-cy was the most potent agonist, with the lowest EC_{50} (16.2 μM) of all agonists used. The rank order of agonist potencies to evoke inward currents in PG neurons was: 3-Br-cy ≫ ACh ≅ cytisine ≫ 5-Br-cy (see Table 1). Taken together, with the very low efficacy of cytisine and the relatively weak partial agonism of 3-bromocytisine at α4 and β2 subunit-containing receptors, this suggests that the current observed here corresponds mainly to α7 nicotinic ACh receptors. The same rank order of potency and relative efficacy of cytisine and its bromo derivatives has been found for human α7 recombinant nAChRs expressed in *Xenopus* oocytes or neuroblastoma cell lines (Houlihan et al., 2001; Slater et al., 2003). In addition, the inward current induced by ACh on PG neurons shows the same time-course of fast inactivation that characterized the response of recombinant human or rat dorsal root ganglion neurons expressing α7 nAChRs (Genzen et al., 2001; Houlihan et al., 2001).

Our electrophysiological recordings suggesting a prominent contribution of α7 subunits agree with the immunohistochemical studies showing the presence of the α7 subunit not only in nerve endings in the carotid body, but also in the somata of PG neurons (Shirahata et al., 1998). Nevertheless, we observed that 59% of the ACh-sensitive neurons (10/17) also respond to 5-Br-cy, suggesting that the current evoked by 5-Br-cy was due to the activation of nAChRs different from the α4β2 subtype, e.g. α4β4. An alternative explanation is that α4β2 nAChRs may differ between cat and human. However, different species have shown nAChRs of identical composition (Itier and Bertrand, 2001; McGehee and Role, 1995), suggesting that other combinations of nAChR subunits (such as α3 and β2) found in the cat PG neurons (Hirasawa et al., 2002; Shirahata et al., 1998) may be functional. It is important to notice that 5 out of 15 neurons which responded to ACh, cytisine and 3-Br-cy did not respond to 5-Br-cy, suggesting that other nAChRs subtypes may exist in cat cultured PG neurons.

In summary, the present results show that PG neurons responded to ACh, cytisine, 3-Br- and 5-Br-cytisine with fast inward currents that desensitized rapidly (during stimuli application) and were reversibly blocked by 1 μM hexamethonium or 10 nM α-bungarotoxin. The order of potency of the agonists on PG neurons was: 3-Br-cytisine ≫ ACh ≅ cytisine ≫ 5-Br-cytisine. Therefore, our results suggest that homomeric α7 nAChRs predominate in cat PG neurons in culture.

4. Experimental procedures

Petrosal ganglia were excised bilaterally from 10 adult cats anesthetized with sodium pentobarbitone (40 mg/kg, i.p.). The ganglia were placed in ice-chilled modified Hanks' solution (Ca^{2+}–Mg^{2+}-free), minced into 15–20 pieces, and enzymatically dissociated under agitation at 38 °C in modified Hanks' solution supplemented with 1 g/L collagenase, 0.5 g/L trypsin and 150,000 U/L DNAse for 30–60 min. The cell suspension was centrifuged for 10 min at 2000 × *g*, and the pellet suspended in F-12 nutrient mixture supplemented with 100 mL/L horse serum, 100 mL/L fetal bovine serum, $NaHCO_3$ (14 mM) and nerve growth factor (15×10^{-6} g/L). The cells were plated onto 35 mm Petri dishes previously coated with poly-L-lysine (0.1 g/L) and maintained at 38 °C in water-saturated, 5% CO_2 in air atmosphere. The culture medium was changed every other day after a 3-day initial lag during which the cultures were left undisturbed. The 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.

The cultures were placed on the stage of an inverted microscope with phase contrast and superfused (flow 1.2 mL/min) at room temperature with Hanks' solution (in mM: NaCl 137, $CaCl_2$ 1.3, $MgSO_4$ 0.8, KCl 5.4, KH_2PO_4 0.4, Na_2HPO_4 0.3, D-glucose 5.6, HEPES 5 and $NaHCO_3$ 4) at pH 7.43 equilibrated with room air. Recordings were made with a patch-clamp amplifier (PC 501-A; Warner Instrument Corp., USA) and 1.5-mm O.D. borosilicate glass pulled electrodes (1–2 MΩ) filled with an intracellular solution (in mM: KCl 135, NaCl 5, $CaCl_2$ 1, EGTA 10, HEPES 10, pH 7.2). Seal formation and membrane breakthrough, from 25 to 65 μm diameter neurons, was carried out in the current-clamp mode and monitored by observing the response to one 2–10 pA depolarizing current step lasting 50 ms.

The currents evoked by ACh, cytisine, 3-Br-Cy and 5-Br-Cy were recorded in the whole cell voltage-clamp mode at a holding potential (V_h) of −60 mV. Liquid junction potential between microelectrodes and bath solution was corrected, and series resistance and capacitive transients were electronically compensated. All agonists were applied for 5–10 s by superfusion under gravity flow from a pipette whose tip was located at about 100–200 μm from the neuron surface. Hexamethonium (1 μM) and α-bungarotoxin (10 nM), in Hanks' solution, were applied for 3 min through a superfusion pipette.

4.1. Purification of cytisine and its analogs

Cytisine was purified from *Sophora secundiflora* seeds using standard methodology. 3-Br-cy and 5-Br-cy were obtained by treating cytisine with slightly more than 1 M equivalent of bromine in acetic acid. The brominated isomers were separated by column chromatography on silica gel, crystallized to homogeneity and characterized by ^1H and ^{13}C NMR.

4.2. Statistical analyses

To compare the effects of ACh, cytisine and its bromo derivatives on the dose–response curves for the inward current (I), pooled data of individual experiments were fitted to the following logistic expression: $I = I_{max} / 1 + (EC_{50} / X)^n$, where I_{max} = maximal current evoked by a given agonist, EC_{50} = agonist concentration that evoked the half-maximal current, X = agonist concentration delivered by the stimulus pipette and n = Hill coefficient. Correlation coefficients for adjusted curves were higher than 0.90 ($P < 0.01$) for all conditions studied.

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