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Airway-related vagal preganglionic neurons express multiple nicotinic acetylcholine receptor subunits

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Abstract

Nicotine acting centrally increases bronchomotor tone and airway secretion, suggesting that airway-related vagal preganglionic neurons (AVPNs) within the rostral nucleus ambiguus (rNA) express nicotinic acetylcholine receptors (nAChRs). In the present study, we examined the three main functionally characterized subtypes of nAChRs in the CNS, the α7 homomeric and α4β2 heteromeric receptors. First, we characterized the expression of these subunits at the message (mRNA) and protein levels in brain tissues taken from the rNA region, the site where AVPNs are located. In addition, double labeling fluorescent immunohistochemistry and confocal laser microscopy were used to define the presence of α7, α4, and β2 nAChRs on AVPNs that were retrogradely labeled with cholera toxin β subunit (CTb), injected into the upper lung lobe ($n=4$) or extrathoracic trachea ($n=4$). Our results revealed expression of all three studied subunits at mRNA and protein levels within the rNA region. Furthermore, virtually all identified AVPNs innervating intrapulmonary airways express α7 and α4 nAChR subunits. Similarly, a majority of labeled AVPNs projecting to extrathoracic trachea contain α7 and β2 subunits, but less than half of them show detectable α4 nAChR traits. These results suggest that AVPNs express three major nAChR subunits (α7, α4, and β2) that could assemble into functional homologous or heterologous pentameric receptors, mediating fast and sustained nicotinic effects on cholinergic outflow to the airways.

Keywords: Rat; Airway control; α7, α4, and β2 nAChR subunits; Vagal preganglionic neurons; Rostral nucleus ambiguus; Retrograde tracing; Cholera toxin β subunit (CTb)

1. Introduction

In humans, as in other mammals, nicotine resorbed from inhaled tobacco smoke may act centrally, to induce a vagally mediated increase in airway smooth muscle tone (Hartiala et al., 1984). Nicotine administered topically to the ventrolateral region of medulla oblongata where the airway-related vagal preganglionic neurons (AVPNs) are located, elevates cholinergic outflow to the airways, inducing bronchoconstriction and airway submucosal gland secretion. These effects could be blocked by centrally administered hexamethonium (Haxhiu et al., 1986; Haxhiu et al., 1991), a general blocker of nicotinic acetylcholine receptors (nAChRs), suggesting the presence of nAChRs on AVPNs.

In rats (Haxhiu et al., 1993), as in other species (reviewed in Kalia, 1987; Jordan, 2001; Haxhiu et al., 2005), the motor preganglionic component of the network innervating the airways arises mainly from the nucleus ambiguus and to a lesser extent from the dorsal motor nucleus of the vagus (DMV). Of these two groups of neurons, AVPNs within the rostral nucleus ambiguus (rNA) play a greater role in generating cholinergic outflow to airway smooth muscle (Haselton et al., 1992), secretory glands, and blood vessels (Haxhiu et al., 2000). Hence, resorbed nicotine and nicotine-like products may act directly on AVPNs, via nAChRs.

nAChRs are pentameric ligand-gated cation channels assembled from a combination of α and β subunits (Cooper et al., 1991; Zoli et al., 1998; Karlin, 2002). In the central nervous system (CNS), there are at least 9α (α2–α10) and three β (β2–β4) subunits. The homomeric assembly of subunits such α7, α8, and α9 has been shown to be

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functional in mammalian cells, whereas α2–α6 and α10 become operational only when coexpressed with β-subunits, as detailed in recent reviews (Berg and Conroy, 2002; Hogg et al., 2003; Dajas-Bailador and Wonnacott, 2004; Gotti and Clementi, 2004).

It is agreed that the two most abundant functionally characterized subtypes of nAChRs within the CNS are the α7 homomeric receptors that bind α-Bungarotoxin with high affinity, called αBgtx-nAChRs, and α4β2 heteromeric nAChRs that express high binding affinity for nicotine, named nAChRs (Lukas and Bencherif, 1992; Gotti and Clementi, 2004). To date, however, subunit composition of nicotinic ligand-gated ion channels expressed by AVPNs has not been investigated. Therefore, in the present studies, we tested the hypothesis that AVPNs innervating extrathoracic trachea and/or intrapulmonary airways express the major nAChR subunits. Our results showed that, in brain tissues of the rNA region, traits of nAChR are present at message and protein levels, and identified AVPNs express α7, α4, and β2 nAChR subunits, which could assemble into functional receptors with homologous or heterologous pentameric channels.

2. Materials and methods

2.1. Animals

In these studies, we used male Sprague–Dawley rats (250–350 g; Harlan, Indianapolis, IN). Male animals were utilized in order to minimize potential physiological changes due to hormonal variations associated with the reproductive cycle. All experimental procedures and protocols were approved by the Howard University Institutional Animal Care and Use Committee.

2.2. RNA extraction and RT-PCR

In the first series of experiments, we employed the reverse transcription-polymerase chain reaction (RT-PCR) to determine whether nAChR mRNAs of α7, α4, and β2 subunits are measurable in the tissues of the rostral ventrolateral medulla oblongata, where AVPNs are located. For these experiments, four adult rats were deeply anesthetized with 100 mg/kg pentobarbital, brains were quickly removed, placed in sterile 0.9% saline solution on ice and then tissue was taken from the rNA region and stored at −80 °C, until used for RNA extraction, as previously described (Zaidi et al., 2005).

Dissected tissues were pooled, homogenized in TRIZOL reagent for RNA extraction (Invitrogen Corporation, CA). The contaminating genomic DNA in the RNA samples was digested using RQ1 RNase free DNase (Promega Corporation, WI). Total cellular RNA was reverse transcribed and amplified with the one-tube and two-enzyme Access RT-PCR System (Promega Corporation, WI). Briefly, 250 ng of total RNA was added to a mixture of reverse transcription/amplification buffer, dNTP mixture, gene specific primer pair, AMV reverse transcriptase and *Tfl* DNA polymerase. Oligonucleotide primers for rat α7, α4, and β2 nAChR subunits were synthesized, corresponding to amino acids sequences selected for each nAChR subunit [α7 (Forward): GTGGAACATGTCT-GAGTACCCCGGAGTGAA, α7(Reverse): GAGTCTG-CAGGCAGCAAGAATACCAGCA; α4 (Forward): GTTCTATGACGGAAGGGTGCAGTGGACA, α4 (Reverse): GGGATGACCAGCGAGGTGGACGGGATGAT; β2 (Forward): ACGGTGTTCCTGCTGCTCATC, β2 (Reverse): CACACTCTGGTCATCATCCTC] as previously published (Lena et al., 1999). β-actin mRNA was used as an internal control to verify the quality of the RNA sample and its subsequent RT-PCR analysis. Primer sequences used were: β-actin (Forward): AACCC-TAAGGCCAACCGTGAAAAG, β-actin (Reverse): CTAGGAGCCAGGGCAGTAATCT. The RT-PCR cycling profiles using a Thermal Cycler (GeneAmp PCR System 9700; Applied Biosystems, CA) were as follows: 1 cycle of reverse transcription at 48 °C for 45 min, 1 cycle at 94 °C for 5 min, 35 cycles at 94 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min, and a final cycle at 72 °C for 7 min. A 10-μl aliquot of each sample was electrophoresed on a 2.0% agarose gel containing 0.5 μg/ml ethidium bromide. The bands of α7, α4, and β2 nAChR subunit mRNAs were presented in parallel with β-actin mRNA levels that were determined from separate RT-PCR reactions.

2.3. Western blotting

The pooled rostral nucleus ambiguus tissue samples of four rats were homogenized in a buffer containing 50 mM Tris pH 7.4, 1% NP40, 0.25% Nadeoxycholate, 150 mM NaCl and 1 mM EDTA using a glass-Teflon homogenizer. The buffer was supplemented with Complete protease inhibitor cocktail (Roche Molecular Biochemicals, IN) and 1 mM Phenylmethylsulfonyl fluoride. The homogenate was rocked on an orbital shaker in the cold room for 15 min. The tissue debris was removed from the homogenate by centrifugation at 14,000×*g* for 15 min. The supernatant, representing the tissue lysate was immediately transferred to a fresh centrifuge tube. All steps involved in the tissue lysis were carried out at 4 °C. An aliquot of the lysate was mixed with an equal volume of 2× Laemmli Sample Buffer (Bio-Rad Laboratories, CA) and the mixture was boiled for 5 min. The protein concentration in the sample was estimated by the Bio-Rad protein assay reagent.

The proteins (50 μg) were separated by size on a 9% SDS-polyacrylamide gel, and transferred to a nitrocellulose membrane in Tris–glycine–methanol buffer containing 25-mM Tris, 192-mM glycine and 20% methanol. For immunoblot detection of nAChRα_4, nAChRα_7, nAChR$\alpha\beta_2$ and β-Actin, membranes were blocked in Tris-buffered saline (TBS) containing 5% nonfat dried milk for 1 h at room