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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 $\alpha 7$ homomeric and $\alpha 4\beta 2$ 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 $\alpha 7$, $\alpha 4$, and $\beta 2$ nAChRs on AVPNs that were retrogradely labeled with cholera toxin *b* 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 $\alpha 7$ and $\alpha 4$ nAChR subunits. Similarly, a majority of labeled AVPNs projecting to extrathoracic trachea contain $\alpha 7$ and $\beta 2$ subunits, but less than half of them show detectable $\alpha 4$ nAChR traits. These results suggest that AVPNs express three major nAChR subunits ($\alpha 7$, $\alpha 4$, and $\beta 2$) 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; $\alpha 7$, $\alpha 4$, and $\beta 2$ 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

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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 9 α ($\alpha 2$ – $\alpha 10$) and three β ($\beta 2$ – $\beta 4$) subunits. The homomeric assembly of subunits such as $\alpha 7$, $\alpha 8$, and $\alpha 9$ has been shown to be functional in mammalian cells, whereas $\alpha 2$ – $\alpha 6$ and $\alpha 10$ 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 $\alpha 7$ homomeric receptors that bind α -Bungarotoxin with high affinity, called α Bgtx-nAChRs, and $\alpha 4\beta 2$ 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 $\alpha 7$, $\alpha 4$, and $\beta 2$ 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 (250–350g; 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 $\alpha 7$, $\alpha 4$, and $\beta 2$ 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 100mg/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, 250ng of total RNA was added to a mixture of reverse transcription/amplification buffer, dNTP mixture, gene specific primer pair, AMV reverse transcriptase and *T7* DNA polymerase. Oligonucleotide primers for rat $\alpha 7$, $\alpha 4$, and $\beta 2$ nAChR subunits were synthesized, corresponding to amino acids sequences selected for each nAChR subunit [$\alpha 7$ (Forward): GTGGAACATGTCTGAGTACCCCGGAGTGAA, $\alpha 7$ (Reverse): GAGTCTGCAGGCAGCAAGAATACCAGCA; $\alpha 4$ (Forward): GTTCTATGACGGAAGGGTGCAGTGGACA, $\alpha 4$ (Reverse):

GGGATGACCAGCGAGGTGGACGGGATGAT; $\beta 2$ (Forward): ACGGTGTTCTGCTGCTCATC, $\beta 2$ (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): AACCTAAGGCCAACCGTGAAAAG, 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 45min, 1 cycle at 94°C for 5 min, 35 cycles at 94°C for 1 min, 60°C for 1 min, 72°C for 1min, and a final cycle at 72°C for 7 min. A 10- μ l aliquot of each sample was electrophoresed on a 2.0% agarose gel containing 0.5 μ g/ml ethidium bromide. The bands of $\alpha 7$, $\alpha 4$, and $\beta 2$ 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 50mM Tris pH 7.4, 1% NP40, 0.25% Nadeoxycholate, 150mM NaCl and 1mM 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 $\times$ 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 $\times$ 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 μ g) 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 nAChR $\alpha 4$, nAChR $\alpha 7$, nAChR $\alpha 2$ and β -Actin, membranes were blocked in Tris-buffered saline (TBS) containing 5% nonfat dried milk for 1h at room temperature with agitation. The membrane was incubated with primary antibodies diluted in TBS (nAChR $\alpha 4$, 1 μ g/ml; nAChR $\alpha 7$, 1 μ g/ml; nAChR $\alpha 2$, 1 μ g/ml and β -Actin, 0.5 μ g/ml) containing 5% nonfat dried milk overnight at 4°C. All antibodies were purchased from Santa Cruz Biotechnology, Inc., CA. The membrane was washed in Tween 20-TBS (TTBS) containing TBS and 0.075% Tween 20 and then incubated for 1 h at room temperature with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin (Bio-Rad Laboratories, CA) diluted 1:15,000 in TBS containing 5% nonfat dried milk. After this incubation, the membranes were washed again in TTBS. The antigen-antibody peroxidase complex was then finally detected by ImmunoStar chemiluminescence kit (Bio-Rad Laboratories, CA) according to the manufacturer's instructions and visualized by exposure to Hyperfilm (Amersham Biosciences, NJ).

2.4. Expression of nAChRs by vagal preganglionic neurons innervating intrapulmonary airways or extrathoracic trachea

For these studies, under pentobarbital anesthesia (50mg/kg, ip), the trachea was injected with cholera toxin β -subunit (CTb), along the tracheal wall ($n = 4$) beginning with the third intercartilaginous space (Haxhiu et al., 1993). To label AVPNs innervating intrapulmonary airways, CTb was injected into the right upper lobe ($n = 4$), as previously described (Hadziefendic and Haxhiu, 1999). After four to five survival days, the animals were anesthetized with pentobarbital (50mg/kg, ip) and perfused through the left ventricle with 300ml of saline, containing 10,000U of heparin. This was subsequently followed by 300ml of 1% paraformaldehyde in 0.1M phosphate buffer (PB, pH 7.4). The brains were removed and

stored in the same fixative for 1h, then transferred to 30% sucrose in 0.1M PB overnight. Transverse sections of the medulla oblongata from pontomedullary border to the decussation of pyramids were cut at 40 μ m using a Bright OTF Cryostat (Hacker Instruments Inc., NJ) and stored in 0.5% sodium azide in 0.1M PB (pH 7.4).

2.4.1. Immunohistochemistry—The expression of specific subunits of nAChRs by identified AVPN innervating the trachea, or the intra-pulmonary airways, was evaluated using sequential double-labeling immunocytochemistry as previously described (Dehkordi et al., 2004; Dehkordi et al., 2005). Briefly, 1-in-5 series of tissue sections of the medulla oblongata were rinsed three times in 0.1M phosphate buffer saline (PBS) at pH 7.4. Tissues were then incubated for 5min in 0.3% Triton-X in PBS and underwent sequential treatment with a series of ethanol and acetone to further increase the penetration of the primary antibody. Endogenous peroxidase activity was quenched before the immunostaining by exposing the sections to 0.1 M PBS containing 0.3% hydrogen peroxide for 30min. Tissues were then placed into a blocking medium containing TNB blocking buffer and 0.1% normal donkey serum (NDS, Santa Cruz Biotechnology, Inc., CA) for 30min. Following these steps, the sections were processed for detection of the α 7, α 4 and/or β 2 subunits of nAChRs using the Tyramide Signal Amplification (TSA) Fluorescence System (PerkinElmer Life Sciences, MA), and then for visualizing CTb labeled AVPNs.

In the first sequence, sections were incubated for 24h at 4°C with the α 7 (rabbit polyclonal, Santa Cruz Biotechnology, 1:10), α 4 (rabbit polyclonal, Santa Cruz Biotechnology, CA, 1:50) or β 2 primary antisera (rabbit polyclonal, Santa Cruz Biotechnology, CA, 1:50). Sections were then washed 3 times, 5min each in 0.1M PBS and incubated with a biotinylated donkey anti-rabbit secondary antibody (Santa Cruz Biotechnology, CA, 1:50) in 0.1M PBS for 1–2h. After washing in PBS (3 $\times$ 5min each), streptavidin conjugated horseradish peroxidase (SA-HRP) was applied to the tissue sections for 30min. The sections were then washed in PBS (3 $\times$ 5min each) and incubated in Fluorophore Tyramide (amplification reagent) for 10min. After the detection of nAChR protein, the tissues were then washed in PBS (3 $\times$ 5min each) and incubated in a 1:10,000 dilution of goat anti-CTb (List Biological Laboratories, Inc., CA) for 48h. Subsequently, the sections were washed in PBS (3 $\times$ 5min ea.) and incubated for 2h in a 1:200 solution of donkey anti-goat IgG conjugated with Alexa Fluor 594 (Texas red, TR; Molecular Probes, Eugene, OR). Finally, the sections were rinsed in PBS (3 $\times$ 5min each), mounted and cover-slipped using Vecta Shield (Vector Laboratories Inc., CA) antifade mounting media.

The control experiments for each of the labeling were done by omission of the primary antibodies, determining whether the secondary antibodies produced falsepositive results. Omission of primary antibodies resulted in the absence of nAChR protein and CTb labeling; thereby demonstrating that no false-positive results were obtained with these reagents.

2.4.2. Fluorescent and laser scanning confocal microscopy—First, slides were viewed with a fluorescent microscope (Olympus AX70, Olympus America, NY) equipped with appropriate filter systems to observe the green (receptor) and red (CTb) fluorescence. Colocalization of nAChR protein with the CTb was identified by viewing the sections and alternating between fluorescence filters. The contrasting immunoprecipitates were readily distinguishable. Sections were also examined, digitized, and indirect immunofluorescence images were collected by use of an Olympus laser scanning confocal microscope. In these experiments, receptor was detected by fluorophore-labeled tyramides (Green) in the presence of biotinylated secondary antibody and SA-HRP as previously described (Dehkordi et al., 2004, 2005). The CTb was detected employing a donkey anti-goat IgG conjugated with Alexa Fluor 594 (Texas red; Molecular Probes, OR). Signals were acquired from the same area of the section, digitized, and stored as tiff files. The Olympus software produced overlay (superimposed) images in which the overlap of the red and green signals generated yellowish

color, thereby indicating the degree to which the staining patterns arising from the different antibodies were codistributed.

2.4.3. Cell counting—A minimum of 8 to 10 rostrocaudal sections of the medulla oblongata (Bregma: -11.60mm to -14.08mm; Paxinos and Watson, 1986) were used for counting of retrogradely labeled vagal preganglionic neurons within the ventrolateral medulla, following CTb injection into the extrathoracic trachea or the upper right lung lobe. We counted the total number of cells containing CTb and the number of neurons that coexpressed CTb and $\alpha 7$, CTb and $\alpha 4$, or CTb and $\beta 2$ subunits, and calculated the percentage of CTb-containing neurons expressing $\alpha 7$, $\alpha 4$ or $\beta 2$ nAChRs.

3. Results

3.1. Expression of $\alpha 7$, $\alpha 4$, and $\beta 2$ subunits at mRNA and protein levels

RT-PCR experiments showed the presence of $\alpha 7$, $\alpha 4$, and $\beta 2$ subunit mRNAs and immunoblotting demonstrated specific receptor proteins in the tissues of the ventrolateral medulla oblongata where AVPNs are located (Fig. 1, left and right panels). These findings formed the foundation for studies on expression of nAChR subunits by identified AVPNs.

3.2. Expression of $\alpha 7$, $\alpha 4$, or $\beta 2$ subunits at single cell level

The expression of $\alpha 7$, $\alpha 4$, or $\beta 2$ was analyzed at the protein level, using specific antibodies that recognize each of these subunits. Adult rat brain sections were incubated with the primary antibody and exposed to the secondary antibody, or were incubated with the secondary antibody alone. Sections treated with a single employed primary antibody and the corresponding secondary antibody gave a consistent pattern of labeling in the brain stem, as previously described (Dehkordi et al., 2004). No cell labeling was found in the absence of the primary antibody of $\alpha 7$, $\alpha 4$ or $\beta 2$.

Using confocal laser microscopy, within the rostral nucleus ambiguus region, the labeling intensity of the analyzed neurons was found to be clearly higher (more than two times) than the background noise. This was observed throughout the entire analyzed region where AVPNs are located. Labeling pattern of $\alpha 7$, $\alpha 4$ and $\beta 2$ receptor subunits was comparable. Immunolabeling of cells within the rNA region resulted in the staining of neurons within the compact and loose portion of the nucleus ambiguus. Labeled cells varied in their morphologies. Extensive $\alpha 7$ labeling was visible throughout cell perikaryon. Labeling of soma surface and specific cellular processes were also observed (Fig. 2). A cellular staining for $\alpha 4$ and $\beta 2$ subunits was not distinguishable from distribution of signals identifying the presence of the $\alpha 7$ subunit.

3.3. Expression of $\alpha 7$, $\alpha 4$, or $\beta 2$ subunits by identified AVPNs

Following injection of CTb into the extrathoracic tracheal wall or intrapulmonary airways and lung parenchyma, majority of retrogradely labeled parasympathetic preganglionic neurons were seen within the rNA: namely the compact portion of the nucleus ambiguus and the area ventral to it. A few neurons were observed in the most rostral part of the dorsal motor nucleus. This labeling pattern correlated well with previous studies in rats, describing vagal preganglionic innervation of the extrathoracic trachea and most distal intrapulmonary airways (Haxhiu et al., 1993; Hadziefendic and Haxhiu, 1999; Perez Fontan and Velloff, 2001).

Double labeling studies showed that virtually every identified vagal preganglionic neuron innervating intrapulmonary or extrathoracic airways expressed $\alpha 7$ like immunoreactivity. Specifically, 151 out of 157 CTb positive neurons, following injections of retrograde tracer into the upper right lung lobe, expressed the $\alpha 7$ nAChR subunit (96%). Similarly, almost all

retrogradely labeled neurons innervating extrathoracic trachea demonstrated strong immunoreactivity for the $\alpha 7$ subunit of nAChRs; 151 out of 161 CTb positive cells (94%) showed strong $\alpha 7$ immunoreactive signal (Fig. 3); differences in expression of $\alpha 7$ subunit by AVPNs providing cholinergic outflow to the intrapulmonary or extrathoracic airways were negligible.

In the same group of rats, studies revealed that 91% (113 out of 123) of vagal preganglionic neurons projecting to the intrapulmonary airways expressed the $\alpha 4$ nicotinic receptor subunit (Fig. 4). However, the number of identified preganglionic neurons innervating extrathoracic trachea that expressed the $\alpha 4$ subunit was somehow lower. Counting of retrogradely labeled cells and double labeled neurons showed that only 38% (67 out of 176) of neurons innervating the extrathoracic trachea express $\alpha 4$ nAChR subunit.

Vagal preganglionic neurons projecting to the extrathoracic trachea were also evaluated for expression of the $\beta 2$ subunit which is known to assemble with $\alpha 4$ to form heteromeric $\alpha 4\beta 2$ nAChRs. Of the 217 CTb positive neurons in the rNA, 74% were immunoreactive for the $\beta 2$ subunit of nAChRs (Fig. 5).

4. Discussion

4.1. Limitations of the techniques

Prior to discussing the findings of the present studies, we will mention the limitations of the techniques used. The results obtained by RT-PCR and Western blotting showed that the all three nAChR subunits are present in the region of the rNA, at mRNA and protein levels. Although RT-PCR and Western blot techniques have high specificity, these findings require careful interpretation. Excised rNA region contains not only vagal preganglionic neurons but also interneurons that lie side by side with those cells that innervate extrathoracic trachea and intrapulmonary airways (Haxhiu et al., 1993). Therefore, observed signals underling specific message and protein subunits may originate from cells other than AVPNs. Hence, these findings were complemented by immunolabeling data.

In addition, the results of immunohistochemistry studies employing subunit specific primary antibodies should be interpreted with caution. A number of control experiments are required to avoid possible nonspecific labeling (Saper and Sawchenko, 2003). In our experiments specificity of the used antibodies was tested using Western blotting, and these antibodies have been previously characterized and successfully used in immunohistochemistry (Schroder et al., 1989; Dominguez del Toro et al., 1994; Ferreira et al., 2001; Dehkordi et al., 2004). In addition in our control experiments, after omitting the primary antisera, no neuronal labeling above the background was observed in the region of rostral nucleus ambiguus or the brain stem sites known to express nAChRs traits at message (Wada et al., 1989) and protein forms (Ferreira et al., 2001; Dehkordi et al., 2004). Hence, we consider that the employed primary antibodies specifically labeled targeted $\alpha 7$, $\alpha 4$, or $\beta 2$ antigen-like molecules.

4.2. Expression of nAChRs by identified AVPNs

The findings of the present study show for the first time that AVPNs express $\alpha 7$, $\alpha 4$ and $\beta 2$ nAChR subunits that could assemble into functional receptors with homologous or heterologous pentameric channels. This could explain nicotine-induced activation of AVPNs, associated with a concomitant increase in airway smooth muscle tone and submucosal gland secretion (Haxhiu et al., 1986; Haxhiu et al., 1991).

4.2.1. The $\alpha 7$ subunit of nAChRs—Fluorescent and laser confocal microscopic analysis of immunolabeling within the rostral nucleus ambiguus region, revealed for the first time that the $\alpha 7$ subunit of nAChRs is postsynaptically expressed by vagal preganglionic neurons

innervating intrapulmonary and extrathoracic airways. Furthermore, our findings indicate that in rats almost every AVPN expresses $\alpha 7$ receptor protein. Clearly, without electron microscopic immunolabeling studies, the precise location of nicotinic receptors cannot be ascertained (Fabian-Fine et al., 2001). However, using confocal laser microscopy, heavy labeling was observed within the membranous elements, including dendritic and postsynaptic cytoplasmic components. Labeling was also observed within perikaryon that could be due to internalized receptor protein. The abundant expression of $\alpha 7$ subunit of nAChRs suggests that nicotinic homomeric receptor channels composed of this subunit could play an important role in regulation of cholinergic outflow to the airways. This assumption is supported by studies using whole-cell recordings. By contrast to the motoneurons of the VII and XII cranial nerves, it was found that the ACh-induced current in vagal preganglionic neurons was mediated by $\alpha 7$ -containing nAChRs. Furthermore, autoradiography revealed dense binding sites for [125 I] α -bungarotoxin in vagal preganglionic neurons, a selective ligand of homomeric $\alpha 7$ -containing nAChRs (Zaninetti et al., 1999). Taken together, these findings indicate the importance of $\alpha 7$ -nAChRs, but do not exclude the possibility of existence of the $\alpha 7$ as a component of a pentameric nAChR consisting of the $\alpha 7$ and $\beta 2$ (Khiroug et al., 2002), or $\alpha 7$ and $\beta 3$ subunits (Palma et al., 1999). This assumption is supported by recent findings showing that the $\alpha 7$ -nAChR subtype that is expressed by the neurons of the rat DMV, is functionally and pharmacologically distinct from homomeric $\alpha 7$ -nAChRs (Ferreira et al., 2001).

As on autonomic ganglionic neurons (Dajas-Bailador and Wonnacott, 2004), the $\alpha 7$ receptor subunit could be present on perisynaptic sites of the AVPNs, and on presynaptic axon terminals. In both human and rats these sites could be involved in transducing effects of ACh and/or choline upon neurotransmitter release and synaptic function (Albuquerque et al., 2000; De Filippi et al., 2005). Activation of perisynaptic nAChRs could cause increase in excitability or depolarization of AVPNs, whereas stimulation of presynaptic receptors localized on axonal terminals innervating vagal preganglionic neurons could modulate the release of several substances, including release of glutamate and facilitation of glutamatergic neurotransmission, as shown in other CNS sites (Aramakis and Metherate, 1998; Alkondon et al., 2003; Jones and Wonnacott, 2004).

4.2.2. Expression of the $\alpha 4$ and the $\beta 2$ subunits of the nAChRs by AVPNs—The results of the present study support previous findings, showing that individual neurons may express multiple classes of nAChRs (as reviewed by Le Novere et al., 2002; Alkondon and Albuquerque, 2004). We found that a majority of identified AVPNs innervating intrapulmonary airways also express the $\alpha 4$ subunit. However, it should be mentioned that only a third of CTb labeled cells, following injection of the retrograde tracer into extrathoracic tracheal wall, showed a clearly detectable $\alpha 4$ immunoreactive signal. The observed difference cannot be fully explained by recent tracer studies, demonstrating at the single cell level, that neurons innervating extrathoracic trachea also provide cholinergic input to intrapulmonary airways (Perez Fontan and Velloff, 2001). Therefore, further investigation, using in situ hybridization technique and physiological approaches, is needed to characterize this phenomenon.

The $\alpha 4$ subunit of nAChRs most often makes a heteromeric assembly with a $\beta 2$ subunit (Flores et al., 1992). Both α - and β -subunits contribute to the agonist sensitivity of neuronal nAChRs (Luetje and Patrick, 1991), which could be influenced by the ratio of $\alpha 4/\beta 2$ (Zwart and Vijverberg, 1998). Expression of $\alpha 4\beta 2$ nAChRs can be affected by multiple factors. For example, chronic exposure of animals to nicotine, or humans to cigarette smoke (Breese et al., 1997; Benwell et al., 1988; Buisson and Bertrand, 2001), leads to upregulation of surface $\alpha 4\beta 2$ nicotinic receptors in a dose- and time-dependent fashion (Ke et al., 1998), which partly results from a decrease in the rate of receptor turnover (Peng et al., 1994). This will enhance the response of $\alpha 4\beta 2$ nAChRs to agonists, leading to depolarization of neurons, via an increase

in the permeability of nicotinic channels to both Na^+ and Ca^{2+} , that in turn could affect synaptic transmission (Matsubayashi et al., 2004). In asthmatic patients this could increase airway responsiveness and cause worsening of pulmonary function even by passive smoking (Dahms et al., 1981; Leuenberger et al., 1994).

4.3. Physiological relevance of nAChR expression by AVPNs

In the CNS, the nAChR system participates in multiple regulatory brain functions (Clementi et al., 2000), including neuronal signaling (reviewed in Tribollet et al., 2001; Dajas-Bailador and Wonnacott, 2004), synaptic transmission and plasticity (reviewed in Albuquerque et al., 2000; Mansvelder and McGehee, 2000), transmitter release (Rowell, 1995; MacDermott et al., 1999; Radcliffe et al., 1999), development (Rossi et al., 2001) and survival of neurons, including spinal cord motor cells (Messi et al., 1997). Furthermore, deficiency of nAChRs is associated with changes in autonomic control. For example, absence of the $\alpha 7$ subunit leads to altered baroreflex responses (Franceschini et al., 2000), whereas a lack of $\beta 4$ (Wang et al., 2003), or $\beta 2$ and $\beta 4$ subunits (Xu et al., 1999) causes multiorgan autonomic dysfunction.

The role of nAChRs in regulating cholinergic outflow to the airways is not fully elucidated. Furthermore, the origin of ACh or choline, as endogenous and selective agonists for nAChRs (Alkondon et al., 1997; Mike et al., 2000) is not clear. CNS cells regulating parasympathetic outflow to the airways are located in the brain stem and in several higher brain regions, containing excitatory and inhibitory neurotransmitters (reviewed in Haxhiu et al., 2005), but no distinct cholinergic cell group that provide monosynaptic inputs to AVPNs was found. It could be postulated that ACh is released by varicosities of passing fibers from pontine structures (Jones, 1990) that are not engaged in synaptic contacts with AVPNs (Haxhiu et al., 1993). ACh released into the extracellular fluid by diffusion will reach nAChRs on AVPNs. This type of neurotransmitter release (Castel et al., 1996) and nonsynaptic transmission (Agnati and Fuxe, 2000) has already been established.

Recently, the role of nAChRs in the transmission of afferent constricting inputs from bronchopulmonary receptors to the nucleus tractus solitarius (nTS) and in the mediation of reflex airway constriction was assessed. In ferrets, it was shown that nTS neurons receiving inputs from the airway sensory system express the nAChR $\alpha 3$ subtype. Whereas activation of these receptors by nicotine increases cholinergic outflow to the airways, their blockade has no significant effect on reflex bronchoconstriction (Ferguson et al., 2000), suggesting that the $\text{AC}\beta$ -nAChR signaling pathway is not required for transmission of information from the airways to the nTS and from the nTS to AVPNs.

Vagal preganglionic neurons innervating the intrapulmonary and extrathoracic airways synthesize ACh (Kc et al., 2004), involved in communication of AVPNs with airway intramural ganglionic cells. ACh can be secreted by soma and dendrites of AVPNs. It was shown that ACh is synthesized and acts as an autocrine growth factor for small cell carcinoma (Song et al., 2003). In analogy, ACh nAChR-induced modulation of vagal preganglionic neuronal discharge could occur in an autocrine manner, through somatodendritic release of ACh, activating perisynaptic receptors, and probably nAChRs localized at glutamatergic nerve terminals innervating AVPNs.

The nAChRs could modulate activity of the AVPNs by mediating effects of endogenous substances such as choline, the tryptophan metabolite kynurenic acid, neurosteroids, and of exogenous compounds, including nicotine and the so-called nicotinic allosteric potentiating ligands and psychotomimetic drugs such as phencyclidine, ketamine (reviewed in Pereira et al., 2002), and antidepressants (Fryer and Lukas, 1999).

In summary, endogenous and exogenous substances may activate $\alpha 7$, $\alpha 7\beta 2$, and/or $\alpha 4\beta 2$ nAChRs expressed by AVPNs and/or those located on the glutamatergic axon terminals innervating AVPNs. While activation of nicotinic receptors expressed by AVPNs will result in depolarization of and in an increase in their firing rate, ACh β - or nicotine-induced activation of presynaptic receptors could facilitate excitatory synaptic neurotransmission (Fig. 6). Hence, blood borne nicotine may trigger bronchoconstriction through activation of presynaptic and/or postsynaptic mechanisms. Further investigation of the functional role of different nAChRs could provide better understanding of the importance of nAChR diversity and the central mechanisms involved in airway hyperresponsiveness induced by repeated exposures to tobacco smoke.

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Fig. 1.

RT-PCR and Western blot analysis of nicotinic acetylcholine receptor mRNA and protein expression in the rostral nucleus ambiguus (rNA) where airway-related vagal preganglionic neurons are located. (A) Schematic representation of a coronal section (Paxinos and Watson, 1986) and (B) the distribution of CTb-labeled AVPNs visualized with rhodamine indicating the location of AVPN within the rNA regions (C), RT-PCR analysis of nicotinic acetylcholine receptor (nAChR) subunits: α_4 , α_7 , β_2 , and β -actin seen at 483, 510, 507, and 638bp, respectively and Western blot analysis of α_4 , α_7 , β_2 , and β -actin, seen at 69, 55, 55, and 41kDa, respectively (Scale bar: 100 μ m).

Fig. 2.

(A) An example of a confocal image of the $\alpha 7$ -nAChR subunit expressed by neurons within the rostral nucleus ambiguus region (rNA). The $\alpha 7$ -nAChR like-immunoreactive product is localized on the cell membrane and dendrites. However, it was also observed in the cytoplasm, suggesting internalization of receptor protein. (B) After omission of primary antibody no labeling of neurons or the fibers was observed (Scale bar: 20 μ m).

Fig. 3. Confocal images of (A), retrogradely labeled airway-related vagal preganglionic neurons (AVPNs) within the rNA, following microinjection of cholera toxin β subunit (CTb) tracer into the upper right lung lobe; (B) $\alpha 7$ -nAChR subunit immunoreactive neurons in the same field. (C) The merged (M) image showing the CTb colocalized with an $\alpha 7$ -nAChR traits. (D) Total number of counted CTb labeled neurons and the number of the double labeled cells (CTb and $\alpha 7$ -nAChR). Scale bar: 20 μ m.

Fig. 4. Confocal images of (A), retrogradely labeled airway-related vagal preganglionic neurons (AVPNs) within the rNA, following microinjection of cholera toxin β subunit (CTb) tracer into the upper right lung lobe; (B) $\alpha 4$ -nAChR subunit immunoreactive neurons in the same field. (C) The merged (M) image showing the CTb colocalized with an $\alpha 4$ -nAChR traits. (D) Total number of counted CTb labeled neurons and the number of the double labeled cells (CTb and $\alpha 4$ -nAChR). Scale bar: 20 μ m.

Fig. 5.

Confocal images of (A), retrogradely labeled airway-related vagal preganglionic neurons (AVPNs) within the rNA, following microinjection of cholera toxin β subunit (CTb) tracer into the extrathoracic trachea; (B) $\beta 2$ -nAChR subunit immunoreactive neurons in the same field. (C) The merged (M) image showing the CTb colocalized with a $\beta 2$ -nAChR traits. (D) Total number of counted CTb labeled neurons and the number of the double labeled cells (CTb and $\beta 2$ -nAChR). Scale bar: 20 μ m.

Fig. 6.

Summary diagram. Possible involvement of acetylcholine (ACh)-nicotinic acetylcholine receptors (nAChRs) signaling pathway in regulating the excitability of AVPNs. ACh released from passing fibers or from AVPNs could reach perisynaptic nAChRs ($\alpha 7$ homologous, $\alpha 7\beta 2$, and/or $\alpha 4\beta 2$ heterologous pentameric channels) causing activation of the cells, or it could activate $\alpha 7$ homomeric receptor expressed by axon terminals innervating AVPNs and facilitate glutamate release and further augment cholinergic outflow to the airways. Nicotine and nicotine-like substances use the same receptors to influence activity of AVPNs.