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Expression of neuronal nicotinic acetylcholine receptors in rat vagal pulmonary sensory neurons

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Abstract

It has been known that cigarette smoke inhalation causes airway irritation and cough, and the effect is caused by both direct and indirect stimulatory effects of nicotine on bronchopulmonary sensory nerves. However, little is known about the expression of nicotinic acetylcholine receptors (nAChRs) in these afferents. In the present study, whole-cell patch-clamp recording and RT-PCR were carried out to examine the expression and function of nAChRs in isolated rat vagal pulmonary sensory neurons that were identified by retrograde labeling with a fluorescent tracer. Patch-clamp recordings demonstrated that application of acetylcholine concentration-dependently evoked an inward current in a subset of pulmonary sensory neurons, which was inhibited by hexamethonium. Application of nicotine or 1,1-dimethyl-4-phenylpiperazinium (DMPP) also activated these neurons, evoking an inward current in voltage-clamp configuration and causing depolarization and action potential in current-clamp recordings. RT-PCR analysis further demonstrated the expression of mRNA encoding for the nAChR subunits $\alpha 4$, $\alpha 5$, $\alpha 6$, $\alpha 7$, $\beta 2$, $\beta 3$ and $\beta 4$, but not $\alpha 2$ and $\alpha 3$ in these neurons.

Keywords

nicotinic acetylcholine receptor; pulmonary sensory neuron; airway irritation; cough

1. Introduction

It has been demonstrated that inhaled cigarette smoke evokes airway irritation accompanied by coughing and bronchospasm in humans, and the responses may have resulted primarily from the stimulatory effect of nicotine on vagal bronchopulmonary C-fibers and rapidly adapting stretch receptors (Lee et al., 1993, 2007). However, whether the stimulatory effect is generated by a direct action of nicotine on these sensory nerves remains to be determined, and there is no direct evidence of the expression of nicotinic acetylcholine receptors (nAChRs) in these pulmonary afferents.

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In the present study, we investigated the expression of neuronal nAChRs in isolated rat vagal pulmonary sensory neurons by using whole-cell patch-clamp recording and RT-PCR. Sensory neurons innervating the lungs and airways were identified by retrograde labeling from the lungs by using the fluorescent tracer, 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate (DiI).

2. Methods

This study was performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health and was also approved by the University of Kentucky Institutional Animal Care and Use Committee.

2.1. Labeling vagal pulmonary sensory neurons

DiI (Invitrogen, Carlsbad, CA) labeling was performed as described in details previously (Kwong and Lee, 2002). Briefly, young adult Sprague-Dawley rats (~160 g) were anesthetized with an intraperitoneal injection of pentobarbital (40 mg/kg) and intubated with a polyethylene catheter (PE 150) with its tip positioned in the trachea above the thoracic inlet. DiI was initially sonicated and dissolved in ethanol, diluted in saline (1% ethanol vol/vol), and then instilled into the lungs (0.2 mg/ml; 0.2 ml $\times$ 2) with the animal's head tilted upward at ~30°.

2.2. Isolation of nodose and jugular ganglion neurons

7–10 days after the DiI labeling, rats were anesthetized with halothane inhalation and decapitated. Nodose and jugular ganglia were extracted and placed in ice-cold Dulbecco's minimal essential medium/F12 (DMEM/F12) solution. Each ganglion was desheathed, cut into ~10 pieces, placed in 0.125% type IV collagenase, and incubated for 1 h in 5% CO₂ in air at 37°C. The ganglion suspension was centrifuged (150 $\times$ g, 5 min) and supernatant aspirated. The cell pellet was resuspended in 0.05% trypsin for 5 min and centrifuged (150 $\times$ g, 5 min); the pellet was then resuspended in a modified DMEM/F12 solution (supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 μ g/ml streptomycin, and 100 μ M MEM nonessential amino acids) and gently triturated with a small bore fire-polished Pasteur pipette. The dispersed cell suspension was centrifuged (500 $\times$ g, 8 min) through a layer of 15% bovine serum albumin to separate the cells from myelin debris. The pellets were resuspended in the modified DMEM/F12 solution, plated onto poly-L-lysine-coated glass coverslips, and then incubated overnight (5% CO₂ in air at 37°C).

2.3. Whole-cell perforated patch-clamp recordings

The coverslip containing the attached cells was placed in a small recording chamber (0.2 ml) that was perfused continuously by gravity-feed with standard extracellular solution (ECS). The chemical stimulants were applied by a pressure-driven drug delivery system (ALA-VM8; ALA Scientific Instruments, Westbury, NY). Recordings were made on pulmonary (DiI labeled nodose/jugular) neurons in the whole-cell perforated patch configuration (50 μ g/ml gramicidin) by using Axopatch 200B/pCLAMP 9.0 (Axon Instruments, Union City, CA). The series resistance was usually in the range of 4–8 M Ω and was not compensated.

The resting membrane potential was held at -70 mV in voltage-clamp mode. The experiments were performed at room temperature ($\sim 22^{\circ}\text{C}$). All chemicals were obtained from Sigma Chemical (St. Louis, MO) and prepared daily with standard ECS.

2.4. RT-PCR

Cytoplasm of ~ 20 individual pulmonary nodose/jugular ganglion neurons was retrieved by aspiration through patch-pipette. The pipette content was expelled into a test tube in which RT-PCR was performed by using Titan One Tube RT-PCT Kit (Roche Applied Science, Indianapolis, IN). A nested PCR was then performed by using the first PCR product as the template. Primers for both the first and nested PCR amplifications were designed to cross exon boundaries to distinguish amplified products from mRNA versus genomic DNA. Primer sequences and predicted product sizes are summarized in Table 1. Reverse transcription was performed at 50°C for 30 min. Cycling conditions for the first PCR were as the following: 1) initial denaturation at 94°C for 3 min; 2) 10 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s, elongation at 68°C for 1 min; 3) 25 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s, elongation at 68°C (45 s – 4 min) cycle elongation of 5 s for each cycle; 4) a prolonged elongation at 68°C for 7 min. Reaction products were subsequently maintained at 4°C until they were used as templates for nested reactions.

The nested PCR was performed as follows by using AccuPrime *Taq* DNA polymerase System (Invitrogen): an initial denaturation at 94°C for 3 min was followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1 min and elongation at 68°C for 2 min. Reaction products were separated on 1% agarose gel in Tris-borate EDTA buffer and visualized with 0.5% ethidium bromide fluorescence.

3. Results

3.1. Patch-clamp recording of nAChR-activated whole-cell responses

A brief application of ACh (3, 10, 30 and $100\text{ }\mu\text{M}$, 2–6 s) evoked an inward current in rat vagal pulmonary sensory neurons; the current amplitude increased as the concentration of ACh was increased (Fig. 1A and C). In a total of 91 neurons tested with both ACh and capsaicin, applications of $100\text{ }\mu\text{M}$ ACh and $1\text{ }\mu\text{M}$ capsaicin evoked an inward current in 36% and 66% of the cells (33 and 60 cells), respectively; in the ACh-sensitive neurons, 64% (21 out of 33 cells) were also activated by capsaicin whereas the remaining 36% were capsaicin-insensitive. The ACh-evoked inward current was almost completely prevented by pretreatment with hexamethonium ($100\text{ }\mu\text{M}$, 5 min), an antagonist of nAChRs ($n = 6$); the inhibiting effect of hexamethonium was reversible after 30 min washing with standard ECS (Fig. 1B).

Nicotine and 1,1-dimethyl-4-phenylpiperazinium (DMPP), two known agonists of nAChRs, also evoked inward current in pulmonary sensory neurons (Fig. 1D and E). When the same concentration ($100\text{ }\mu\text{M}$) of nicotine, DMPP and ACh were applied in the same neurons, the inward currents evoked by nicotine and DMPP showed similar amplitude, whereas the ACh-evoked current was considerably smaller in all four neurons tested (Fig. 1D and F). The sensitivities to nicotine varied among different pulmonary sensory neurons; and the whole-

cell current evoked by nicotine exhibited distinct patterns in different neurons. For example, the inward current evoked by 100 μ M nicotine displayed relatively slow inactivation in 10 of 15 neurons (e.g., Fig. 1D), and rapid inactivation in the remaining cells (e.g., Fig. 1G). Furthermore, our current-clamp recordings demonstrated that nicotine, DMPP and ACh evoked depolarization of membrane potential and generation of action potentials in these neurons (e.g., Fig. 1D, lower panel).

In a subset of eight neurons, we have tested the time course of recovery from nicotine (100 μ M, 3–4 s)-induced desensitization. After the control response to nicotine was established, neurons were bathed in the standard ECS for progressively shortening intervals before they were again challenged with the same nicotine solution. Our results showed that 60–120 s was required for a complete recovery from nicotine-induced desensitization (e.g., Fig. 1H).

3.2. Expression of nAChR mRNA in rat vagal pulmonary sensory neurons

RT-PCR analysis revealed that pulmonary sensory neurons express mRNA for the subunits α 4, α 5, α 6, α 7, β 2, β 3 and β 4 of the nAChR, whereas α 2 and α 3 subunits were not detected (Fig. 2). Results were confirmed in four separate trials using DiI labeled nodose/jugular ganglion neurons collected from four different rats. The primers for all the nAChR subunits were successfully tested with our positive control: 150 ng of total RNA extracted from the brain of an adult Sprague-Dawley rat of similar age; in contrast, control reactions that did not contain RNA template showed no amplification products (data not shown).

4. Discussion

Results from our patch-clamp recordings have demonstrated that ACh concentration-dependently evoked an inward current in a subset of rat vagal pulmonary sensory neurons, and this response was almost completely blocked by hexamethonium, indicating the involvement of activation of nAChRs. Our data showed that 36% of the pulmonary sensory neurons were activated by 100 μ M ACh; this percentage is similar to that found in our previous study, which showed that 100 μ M nicotine evoked a pronounced and abrupt Ca^{2+} transient in 34% of the pulmonary sensory neurons tested (Xu et al., 2007). In addition, our patch-clamp recordings have demonstrated that nicotine, DMPP and ACh could cause the membrane depolarization and action potential firing in these neurons. Furthermore, our results have shown that the inward current evoked by nicotine displayed clearly different inactivation patterns, which may suggest activation of different nAChR subtypes (Gotti et al., 1997; Genzen et al., 2001; Gotti and Clementi, 2004; Jensen et al., 2005).

Neuronal nAChRs are pentameric ligand-gated ion channels formed by five transmembrane subunits (Kalamida et al., 2007). To date, nine α (α 2– α 10) and three β (β 2– β 4) neuronal subunit genes have been cloned, and these subunits assemble in numerous combinations to form functional nAChR subtypes (Jensen et al., 2005). It is now known that the α 2– α 6 and β 2– β 4 subunits form heteromeric nAChR complexes; α 7, α 8 and α 9 can form homomeric receptor complexes and α 10 form a functional nAChR together with α 9; whereas α 8 has been found exclusively in chicken either as homopentamer or in combination with α 7 (Jensen et al., 2005; Kalamida et al., 2007). Receptor subunit composition is the principal determinant of nAChR properties, including agonist and antagonist selectivity profiles,

activation and desensitization kinetics and cation permeability (Genzen et al., 2001; Jensen et al., 2005); for example, when expressed with the $\beta 2$ subunit, the $\alpha 2$, $\alpha 3$, $\alpha 4$ and $\alpha 6$ subunits all form functional channels that differ in their channel open time, single channel conductance and agonist/antagonist sensitivity (Gotti et al., 1997), whereas expression of the homomeric ($\alpha 7$ – $\alpha 9$) nAChRs shows dramatically larger Ca^{2+} permeabilities and faster desensitization rates than those of the heteromeric nAChRs (Gotti and Clementi, 2004; Jensen et al., 2005). Although the expression and function of neuronal nAChRs have been studied more extensively in the central nervous system, the expression of various neuronal nAChR subunits has also been reported in the peripheral sensory system such as trigeminal and dorsal root ganglia (Keiger et al., 2000; Genzen et al., 2001; Rau et al., 2005). In the present study, our RT-PCR analysis revealed that rat vagal pulmonary sensory neurons express mRNA for the nAChR subunits $\alpha 4$, $\alpha 5$, $\alpha 6$, $\alpha 7$, $\beta 2$, $\beta 3$ and $\beta 4$, whereas $\alpha 2$ and $\alpha 3$ subunits were not detected, and $\alpha 9$ and $\alpha 10$ subunits were not measured. To our knowledge, this is the first report of expression of mRNA encoding for neuronal nAChR subunits in vagal pulmonary sensory nerves.

It should be pointed out that our RT-PCR results were based on the analysis of mRNA expression from a pool of multiple neurons. Therefore, we do not have the definite evidence to prove the existence of specific subtypes of nAChRs and their subunit compositions in individual pulmonary sensory neurons. To better understand the role of nAChRs in regulating the excitability of pulmonary sensory nerves, further study with the employment of additional technical approaches such as single-cell RT-PCR and use of more specific agonists/antagonists for different subtypes of neuronal nAChRs will be required.

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Fig. 1.

ACh, nicotine and DMPP-evoked whole-cell responses in rat vagal pulmonary sensory neurons. (A) Representative records illustrating that ACh (3, 10, 30 and 100 μM , 3 s duration) concentration-dependently evoked an inward current in a pulmonary sensory neuron (jugular; capacitance: 24.9 pF). (B) Representative records illustrating that ACh (100 μM , 2 s)-evoked inward current was reversibly inhibited by hexamethonium (Hex; 100 μM , 5 min) in a nodose neuron (19.6 pF). (C) Group data showing the inward current evoked by increasing concentration of ACh (3–100 μM , 2–6 s). (D) Representative records illustrating whole-cell responses (upper panel: voltage-clamp recording; lower panel: current-clamp recording) to nicotine (Nic; 10, 30 and 100 μM , 4 s), DMPP (10, 30 and 100 μM , 4 s) and ACh (100 μM , 4 s) in a single nodose neuron (27.2 pF). Insets, action potentials shown on a larger scale. (E) Group data showing the inward current evoked by different concentrations of nicotine (3–100 μM , 3–6 s). (F) Comparison of the inward currents evoked by the same concentration (100 μM , 3–4 s) of nicotine, DMPP and ACh recorded from the same neurons. (G) Representative records illustrating a different phenotype of inward currents evoked by nicotine (10, 30 and 100 μM , 6 s) in a jugular neuron (28.6 pF). (H) Representative records illustrating the inward currents evoked by 100 μM nicotine (3 s) at different intervals between two consecutive challenges in a nodose neuron (21.2 pF).

Fig. 2.

RT-PCR analysis of nAChR mRNA in rat vagal pulmonary sensory neurons. Cytoplasm of 20 isolated DiI-labeled nodose/jugular ganglion neurons was collected into a single PCR tube. One-step RT-PCR and a nested PCR were carried out to detect the mRNA for nAChR subunits. The nested PCR products were run on a 1% agarose gel and stained with ethidium bromide. PCR product sizes matched predictions made from known subunit sequences shown in Table 1. M, 100 bp DNA marker.

Table 1

PCR primer sequences

Subunit	Accession no.	Primer for the 1 st PCR	Size (bp)	Primers for the nested PCR	Size (bp)
$\alpha 2$	NM_133420	5'-AGCCAGCTCTTGGTAGTCCA-3'	830	5'-TGACCAACCAATGCTGGGTA-3'	343
		5'-AGCACAGTGAGGCAGGAGAT-3'		5'-GTCCTCTCCATCTGCTCCAG-3'	
$\alpha 3$	NM_052805	5'-TGCTCAGCTGGTGAAGGTG-3'	977	5'-CCAGATCATGGAAACCAACC-3'	483
		5'-TTCCCTGCAGCTTTTGGAGT-3'		5'-CGACGGATGTACAGCGAGTA-3'	
$\alpha 4$	NM_024354	5'-CCAGATGATGACAAACCAACG-3'	729	5'-CAGGAGTGGCACGACTACAA-3'	449
		5'-ATGACGATGGAGAGGGTGAC-3'		5'-CGGATGATGAAGGCATAGGT-3'	
$\alpha 5$	NM_017078	5'-AATTCGGCTCGTGGACATAC-3'	1063	5'-CAGTCGTGAAAGAGAAACGA-3'	386
		5'-GATGCAGATGCTAGGCCCTTC-3'		5'-GTGCAGGCAGACAATGTGTT-3'	
$\alpha 6$	NM_057184	5'-TCGTGCTGTGATCTTGCAG-3'	973	5'-GACTGACAGTTCGGTTTCC-3'	451
		5'-GATGTGGATGGATGGTCTC-3'		5'-GAAAGTCACCGACGGCATTAT-3'	
$\alpha 7$	NM_012832	5'-GGAGGCTGTACAGGAGCTG-3'	947	5'-GGCTGTACAAAGGAGCTGGTC-3'	415
		5'-AAACCATGCACACCAAGTTCA-3'		5'-AGTTTGCACTGCTGCACATC-3'	
$\beta 2$	NM_019297	5'-TGCTAGCACTCACGGTGTTC-3'	801	5'-TCAAGGTGGTCTTCCTGGAG-3'	418
		5'-ATGTGTGAACGGAGGTGTGA-3'		5'-ACGGTCCCAAAGACACAGAC-3'	
$\beta 3$	NM_133597	5'-TCTGCGCTTGAAAGGAAAAAT-3'	752	5'-TTCCTGGGTGACTCTTACGG-3'	390
		5'-GGCGTCTCAGGACAAAAAGAG-3'		5'-ACGGTTCCACTGGACTTTCAC-3'	
$\beta 4$	NM_052806	5'-CTCCTGAACAAAAACCCGGTA-3'	844	5'-ACAGATCATGACCACCAAGCA-3'	640
		5'-ACACAGTGGTGACGATGGAA-3'		5'-CACGATCTTGGAGATGAGCA-3'	