Original Paper

Nicotine Elevated Intracellular Ca^{2+} in Rat Airway Smooth Muscle Cells via Activating and Up-Regulating $\alpha 7$ -Nicotinic Acetylcholine Receptor

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Key Words

Nicotinic acetylcholine receptors • Intracellular calcium • Airway smooth muscle cells • Nicotine

Abstract

Background: Chronic obstructive pulmonary disease (COPD) is characterized by airway remodeling with airway smooth muscle (ASM) hypertrophy and hyperplasia. Since tobacco use is the key risk factor for the development of COPD and intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) plays a major role in both cell proliferation and differentiation, we hypothesized that nicotinic acetylcholine receptor (nAChR) activation plays a role in the elevation of $[\text{Ca}^{2+}]_i$ in airway smooth muscle cells (ASMCs). **Methods:** We examined the expression of nAChR and characterized the functions of $\alpha 7$ -nAChR in ASMCs. **Results:** RT-PCR analysis showed that $\alpha 2-7$, $\beta 2$, and $\beta 3$ -nAChR subunits are expressed in rat ASMCs, with $\alpha 7$ being one of the most abundantly expressed subtypes. Chronic nicotine exposure increased $\alpha 7$ -nAChR mRNA and protein expression, and elevated resting $[\text{Ca}^{2+}]_i$ in cultured rat ASMCs. Acute application of nicotine evoked a rapid increase in $[\text{Ca}^{2+}]_i$ in a concentration-dependent manner, and the response was significantly enhanced in ASMCs cultured with 1 μM nicotine for 48 hours. Nicotine-induced Ca^{2+} response was reversibly blocked by the $\alpha 7$ -nAChR nicotinic antagonists, methyllycaconitine and α -bungarotoxin. Small interfering RNA suppression of $\alpha 7$ -nAChR also substantially blunted the Ca^{2+} responses induced by nicotine. **Conclusion:** These observations suggest that nicotine elevates $[\text{Ca}^{2+}]_i$ in ASMCs through $\alpha 7$ -nAChR-mediated signals pathways, and highlight the possibility that $\alpha 7$ -nAChR can be considered as a potential target for the treatment of airway remodeling.

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Introduction

It is well documented that tobacco use is the most important risk factor for the development of chronic obstructive pulmonary disease (COPD), which is characterized with profound airway remodeling, including airway smooth muscle hypertrophy and hyperplasia [1–4]. However, the role of the major active component of tobacco, nicotine, in COPD is still unclear. The primary molecular target of nicotine is the nicotinic acetylcholine receptors (nAChRs), which are ligand-gated channels composed of homo- or heteropentamer of various subunits ($\alpha 1$ – $\alpha 10$; $\beta 1$ – $\beta 4$) and operate as cation entry pathways across the plasma membrane [5–7]. Nicotine interacts with various nAChR subtypes with different affinities (1 to 130 μM) [8, 9].

Activation of nAChR elicits Ca^{2+} response in part through direct Ca^{2+} permeation via the receptor channel [10]. In addition, nAChR-mediated membrane depolarization can activate voltage-dependent Ca^{2+} channels (VDCCs) to further augment Ca^{2+} signals generated by nAChRs [11–13]. These two mechanisms are complementary physiologically, such that Ca^{2+} entry through the inwardly rectifying nAChR channels is predominant under either resting or hyperpolarized conditions, whereas Ca^{2+} influx through VDCCs occurs at more depolarizing potentials (> -40 mV) [13–15]. In addition to extracellular Ca^{2+} entry, Ca^{2+} release from intracellular stores may also play a crucial role in defining nAChR mediated Ca^{2+} responses. Activation of Ca^{2+} stores following stimulation of nAChR contributes to the long-lasting Ca^{2+} signals neuronal cells [12, 16–20]. Specific nAChR subtypes are associated with defined Ca^{2+} mobilization pathways. For example, nAChRs that contain $\alpha 3$ and/or $\beta 2$ subunits in brain and ganglionic preparations are associated predominantly with Ca^{2+} signals mediated by depolarization and activation of VDCCs [12, 18, 19], whereas $\alpha 7$ -nAChRs generate robust Ca^{2+} transients which reflect Ca^{2+} entry through the nAChR and Ca^{2+} -induced- Ca^{2+} release (CICR) [12, 18, 21]. Functional coupling between $\alpha 7$ -nAChRs and ryanodine receptors has been shown in hippocampal astrocytes, where $\alpha 7$ -nAChR-mediated Ca^{2+} signals arise primarily from CICR through ryanodine receptors [21, 22].

$\alpha 7$ -nAChR subunits form functional homo-pentameric nAChR which has a much higher Ca^{2+} permeability than other nAChRs [23–25]. $\alpha 7$ -nAChR is activated by agonists such as choline and nicotine [23, 26] and blocked by antagonists such as α -bungarotoxin and methyllycaconitine (MLA) [27, 28]. Furthermore, $\alpha 7$ -nAChR exists in a variety of cell types including neuron [23], bronchial epithelial cells [29], aortic endothelial cells [29], macrophage [29], and artery smooth muscle cells (ASMCs) [30]. However, the expression and function of $\alpha 7$ -nAChR in the airway smooth muscle cells remain unclear.

In the present study, we tested the hypothesis that nicotine modulates ASMC functions through the activation of nAChRs. We sought to identify the nAChR subtypes expressed in rat ASMCs and characterize the effects of chronic and acute nicotine exposure on Ca^{2+} mobilization in ASMCs. Our results herein demonstrated that (1) multiple functional nAChRs are expressed in rat ASMCs; (2) nicotine exposure upregulates $\alpha 7$ -nAChR expression and function; and (3) the biological effects of nicotine may result from $\alpha 7$ -nAChR mediated increase in $[Ca^{2+}]_i$. These results suggest that $\alpha 7$ -nAChRs may be the major target of nicotine in ASMCs, and its upregulation during nicotine exposure may lead to elevated $[Ca^{2+}]_i$, providing a novel mechanism for airway remodeling.

Materials and Methods

Rat ASMC isolation and culture

Similar to the isolation of pulmonary arterial smooth cells described previously [31], bronchi were dissected from lungs of male Sprague Dawley rats (body wt 300–500 g). The epithelium was removed by rubbing the luminal surface with a cotton swab. The bronchi were allowed to recover for 30 min in cold (4°C) physiological salt solution (PSS) that contained (in mM) 130 NaCl, 5 KCl, 1.2 Mg Cl_2 , 10 HEPES, and 10 glucose. It was followed by 20 min in reduced- Ca^{2+} PSS (20 μM $CaCl_2$) at room temperature. The tissue

was then digested at 37°C for 20 min in reduced- Ca^{2+} PSS containing collagenase (type I, 1,750 U/ml), papain (9.5 U/ml), bovine serum albumin (2 mg/ml), and dithiothreitol (1 mM). After resuspension and trituration of the digested tissue, cells were plated onto 25-mm coverslips (for fluorescent microscopy) or 10-cm petri dishes (for molecular biological measurements) and incubated for 3–6 days in smooth muscle growth medium 2 (Clonetics, Walkersville, MD) containing 5% serum in a humidified atmosphere of 5% CO_2 -95% air at 37°C. Twenty-four hours before an experiment, the concentration of serum in culture media was decreased to 0.5% to stop cell growth.

Cellular purity of the cultures was assessed by examining morphological appearance under phase-contrast microscopy and immunofluorescence staining for α -actin under fluorescence microscopy. For the latter, ASMCs were treated with a primary monoclonal antibody raised against smooth muscle α -actin (Sigma, St. Louis, MO), Cy3-conjugated secondary antibody (excitation λ = 550 nm, emission λ = 570 nm; Jackson ImmunoResearch, West Grove, PA), and a nuclear stain (YO-PRO-1, excitation λ = 488 nm, emission λ = 509 nm; Molecular Probes, Eugene, OR). Cells were examined under a Zeiss LSM-510 inverted laser-scanning confocal fluorescence microscope with a Zeiss Plan-Neofluor $\times 40$ oil immersion objective (Atlanta, GA). For each determination, we inspected at least 1,000 cells in at least 40 randomly selected fields. Coverslips not exposed to the primary antibody, but otherwise treated similarly, served as controls.

Measurement of intracellular Ca^{2+}

As previously described [32], after incubation with 5 μM fura-2 (Molecular Probes) for 60 min at 37°C under an atmosphere of 5% CO_2 -95% air, coverslips with rat ASMCs were mounted onto a closed polycarbonate chamber clamped in a heated aluminum platform (PH-2; Warner Instrument, Hamden, CT) on the stage of a Nikon TSE 100 Ellipse inverted microscope (Melville, NY). The chamber was perfused at 0.5 ml/min with Krebs-Ringer bicarbonate (KRB) solution, which contained (in mM) 118 NaCl, 4.7 KCl, 0.57 $MgSO_4$, 1.18 KH_2PO_4 , 25 $NaHCO_3$, 2.5 $CaCl_2$, and 10 glucose. KRB solution was equilibrated with 16% O_2 -5% CO_2 at 38°C in heated reservoirs and led via stainless steel tubing and a manifold to an in-line heat exchanger (SF-28, Warner Instrument), which rewarmed the perfusate just before it entered the cell chamber. The temperature of the heat exchanger and chamber platform was controlled by a dual-channel heater controller (TC-344B, Warner Instrument). This system maintained temperature at 37°C and oxygen tension at 112 ± 2.0 mmHg at the coverslip. After perfusing the chamber for 10 min to remove extracellular dye, ratiometric measurement of fura-2 fluorescence at 60-s intervals was performed using a collimated light beam from a xenon arc lamp filtered by interference filters at 340 and 380 nm and focused onto rat ASMCs visualized with a $\times 20$ fluorescence objective (Super Fluor 20; Nikon, Torrance, CA). Light emitted from the cells at 510 nm was returned through the objective and detected by a cooled charge-coupled device imaging camera. An electronic shutter (Vincent Associates, Rochester, NY) was used to minimize photobleaching. Protocols were executed, and data were collected on-line with InCyte software (Intracellular Imaging, Cincinnati, OH). $[Ca^{2+}]_i$ was calculated from fura-2 fluorescence ratios (F340/F380) using linear regression between adjacent points on a calibration curve generated by measuring F340/F380 in at least seven calibration solutions with $[Ca^{2+}]$ between 0 and 610 nM (Molecular Probes). Because the behavior of fura-2 in solution may differ from that in cells, calculated $[Ca^{2+}]_i$ should be considered as an estimate of the actual $[Ca^{2+}]_i$.

RNA isolation and measurement by RT-PCR and real-time qPCR

Total RNA was prepared from rat ASMCs, as previously described [33]. Briefly, samples were placed in cold TRIzol reagent (50–100 mg tissue/ml) and homogenized at 20,000 rpm (Ultra-Turrax T-25). After incubation at 30°C for 5 min and addition of chloroform (0.2 ml/ml TRIzol), samples were centrifuged at 8,000 g at 4°C for 15 min. The upper aqueous phase of the supernatant was mixed with 100% isopropanol (0.5 ml/ml TRIzol), incubated at 30°C for 10 min, and recentrifuged at 4°C and 12,000 g for 10 min. The clear gel-like RNA precipitate was washed with 1 ml of 70% alcohol, dissolved in diethyl pyrocarbonate water (1 $\mu g/\mu l$), and stored at -70°C. The quality of the RNA isolate was determined from the ratio of absorbance at 260 nm to that at 280 nm (>1.7) and the integrity of the samples was judged by examining 28S and 18S ribosomal RNA bands appearing on electrophoresis of denatured RNA samples through a 1% agarose formaldehyde gel.

RT was performed with the First-Strand cDNA Synthesis kit (Pharmacia Biotech, Austin, TX), as previously described [27]. The resultant cDNA was subjected to PCR by adding 4 μl of the First Strand cDNA reaction mixture to a 50- μl PCR reaction mixture, which consisted of 1 pM of each PCR primer, 10 mM

Table 1. nAChR primer sequence for RT-PCR and real-time PCR

Gene	Accession Number	Source	Primer Pair Sequence(sence/antisence)	Product Size(bp)	Location in sequence
$\alpha 1$ -nAChR	NM024485	Rat	5'-TTGCGTCCATAATAATCAC-3' 5'-GAGGGAGGCTTAGTTTCAG-3'	483	978-1460
$\alpha 2$ -nAChR	NM 133420	Rat	5'-GCTCATCTCTCCTGCCTCAC 3' 5'-TCTTCTTCTCTCCTCCTTCTCCTCTG-3'	433	1066-1498
$\alpha 3$ -nAChR	NM052805	Rat	5'-GAGTGGCGAGTGGGCTAT-3' 5'-CGAGGTGGAAGGAATGGT-3'	285	591-876
$\alpha 4$ -nAChR	NM024354	Rat	5'-GCCAAGATTGACTTAGTGAGC-3' 5'-GGTTGATGGTGTAGAATAGCG-3'	189	574-763
$\alpha 5$ -nAChR	NM017078	Rat	5'-ATCCTGGCAAACCTACC-3' 5'-AACAGGGCAAAGTGGAGC-3'	231	1678-1908
$\alpha 6$ -nAChR	NM057184	Rat	5'-CCCACTACAACCGTTCA-3' 5'-AAGTCACCGACGGCATT-3'	262	306-507
$\alpha 7$ -nAChR	NM012832	Rat	5'-GCAAAGAGCCATACCCAG-3' 5-CAGCAAGAATACCAGCAGAG-3	124	638-762
$\beta 1$ -nAChR	NM012528	Rat	5'-ACCAACTACCCGAACCAC-3' 5'-TTCCCAGAAACAAGAACAAG-3'	496	1173-1668
$\beta 2$ -nAChR	NM019297	Rat	5'-TCATCATTCGTCGCAAAC-3' 5'-TAGCAGCACAGAAATACAAAGT-3'	140	868-1007
$\beta 3$ -nAChR	NM133597	Rat	5'-TAAGGTCTTCTTGGTTCT-3' 5'-GGAGTGCGTCTTCGTGTT-3'	102	191-292
$\beta 4$ -nAChR	NM052806	Rat	5'-CACAACTGCCACCTTC-3' 5'-GCTGACACCTCTAATGCT-3'	294	1012-1305

Tris_HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 200 μ M each dNTP, and 2 units *Taq* DNA polymerase. Primer sequences specific for rat nAChRs, or β -actin were designed using Primer 5 software and are listed in Table 1. The fidelity and specificity of these oligonucleotides were confirmed using the Basic Local Alignment Search Tool program. cDNA was amplified in a DNA thermal cycler (model 2400 GeneAmp PCR System; Perkin-Elmer, Foster City, CA). The mixture was annealed at 56–58°C (1 min), extended at 72°C (2 min), and denatured at 94°C (1 min) for 31–35 cycles. It was followed by a final extension at 72°C (10 min) to ensure complete product extension. The PCR products were electrophoresed through a 1.5% agarose gel, and the amplified cDNA bands were visualized by ethidium bromide staining.

cDNA was quantified by real-time qPCR using QuantiTect SYBR Green PCR Master Mix (Qiagen) in a iCyclerIQ real-time PCR detection system (Bio-Rad) using the following conditions: 95°C for 15 min and 45 cycles, each at 94°C for 15 s, 56°C for 20 s, and 72°C for 20 s. The volume of each real-time qPCR reaction mixture was 25 μ l containing 300 nM forward and reverse primers and cDNA template from 50 ng RNA. Identity of the qPCR products was confirmed by 1) a single sharp peak in the melting curve performed after cDNA amplification; 2) a single band of the expected size resolved by agarose gel electrophoresis; and 3) the correct DNA sequence. Melting curves were performed at 95°C for 1 min and 55°C for 1 min, followed by 80 increments of 0.5°C at 10-s intervals. Real-time qPCR detection threshold cycle values were generated by iCyclerIQ software. Relative concentrations of each transcript were calculated using the Pfaffl method [34]. Mean efficiencies of amplification for each gene were used to quantify expression of $\alpha 7$ -AChR relative to β -actin in the same sample.

Protein isolation and determination by Western blotting

Primary cultures of ASMCs were washed with phosphate-buffered saline and homogenized in HEPES buffer at 20,000 rpm (Ultra-Turrax T-25), total protein concentration in the homogenate was determined by the BCA protein assay (Lowry method) as described previously [35]. Homogenate proteins were separated by SDS-PAGE calibrated with prestained protein molecular weight markers (Bio-Rad, Hercules, CA). Separated proteins were transferred to nitrocellulose membranes (Hybond-C, Bio-Rad). After blocked with 5% nonfat dry milk in Tris-buffered saline and 0.1% Tween 20, membranes were incubated with affinity-purified polyclonal antibodies specific for $\alpha 7$ -AChR (Santa Cruz Biotechnology, Santa Cruz, CA). The membranes were then washed and incubated with anti-rabbit horseradish peroxidase-conjugated IgG for 1 hour, and an enhanced luminol-linked chemiluminescence detection system (Pierce Biotechnology, Rockford, IL) was used to detect the bound antibody.

Fig. 1. Identification of ASMCs. (A) Phase-contrast image ($\times 100$) of rat airway smooth muscle cells (ASMCs) cultured for 5 days. (B) Fluorescence immunocytochemical image ($\times 200$) of smooth muscle α -actin (shown in red) and the nuclear marker YO-PRO-1 (shown in green) in ASMCs culture for 5 days.

RNA Interference Experiments

We selected two sites in the $\alpha 7$ -AChR mRNA sequence as siRNA targets based on principles described previously [36]. The targeted $\alpha 7$ -AChR sequences, based on which the siRNAs were chemically synthesized by Invitrogen (Carlsbad, CA), were 5'-UCUCCUGAACUGGUGUGCAUGGUUU-3' (siRNA-1) and 5'-AAACCAUGCACACCAGUUCAGGAGA-3' (siRNA-2). The negative control siRNAs were purchased from Invitrogen. *In vitro* transfections of ASMCs with 10 μ g/ml siRNA or control sequence were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) following manufacturer's protocols. The efficiency of $\alpha 7$ -AChR suppression was determined by Western blot, and the Ca^{2+} fluorescence experiments were performed 72 hours after siRNA transfection.

Drugs and materials

The following drugs used in this study were obtained from Sigma: nicotine, methyllycaconitine (MLA), α -bungarotoxin. Activated pervanadate solution was prepared freshly for each experiment and made by mixing a stock solution of vanadate with H_2O_2 in an equal molar ratio. Fura-2-AM (Molecular Probes) was prepared on the day of the experiment as a 2.5 mM stock solution in dimethyl sulfoxide (DMSO).

Statistical analysis

Data are expressed as means $\pm$ SEM, where n is the number of experiments performed, and the number of cells in each experiment ranged between 20 and 40, as indicated in results section and the figure legends. Statistical comparisons were performed using Student's *t*-test. Differences were considered to be significant when $P < 0.05$.

Results

Characteristics of ASMCs

ASMCs cultured from rat pulmonary bronchi were phase bright and assumed a spindle-shaped appearance with cytoplasmic projections extending from a larger central area that contained the nucleus (Fig. 1A). Immunostaining for smooth muscle α -actin was positive in $>95\%$ of cells (Fig. 1B). None of the cells were positive when staining was performed without the primary anti- α -actin antibody.

Nicotine elevated baseline intracellular Ca^{2+} in ASMCs

It has been reported that nicotine treatment raises resting $[Ca^{2+}]_i$ in other cells [12, 37], but whether nicotine exerts its effect in ASMCs is unclear. To examine this possibility, ASMCs were cultured in the presence of nicotine. As depicted in Fig. 2B, a noticeable increase in baseline $[Ca^{2+}]_i$ was observed in nicotine (100 μ M) treated culture cells after exposure periods of 24 to 72 hour. The nicotine effect on resting $[Ca^{2+}]_i$ was concentration-dependent, with significant induction at concentrations from 10^{-6} to 10^{-4} M after 48 hours (Fig. 2A). The most robust and consistent effect was seen at 10^{-4} M. A higher concentration of nicotine (10^{-3} M) caused a decline in the change of baseline $[Ca^{2+}]_i$, possibly due to inactivation of

Fig. 2. The effect of nicotine on the baseline $[Ca^{2+}]_i$ of ASMCs. (A) Nicotine increased baseline $[Ca^{2+}]_i$ in a concentration-dependent fashion. ASMCs were cultured with increasing concentrations of nicotine (10^{-7} to 10^{-3} M) for 48 hours. (B) Nicotine increased baseline $[Ca^{2+}]_i$ in a time-dependent fashion. ASMCs were cultured with 10^{-4} M nicotine for the indicated hours. *Significant difference from untreated control condition ($P < 0.05$).

Fig. 3. Identification of nAChR expression and the effect of chronic nicotine exposure on $\alpha 7$ -nAChR mRNA expression in cultured rat ASMCs. (A) $\alpha 1$ -7 and $\beta 1$ -4 nAChR mRNA expression in cultured rat ASMCs detected by RT-PCR (n=3 experiments in 3 animals). RT-PCR

was carried out using specific primers shown in Table 1. (B) RT-PCR analysis of nicotine stimulation of $\alpha 7$ -nAChR mRNA expression in ASMCs cultured for the indicated period of time in the absence or presence of $10 \mu\text{M}$ nicotine. (C) Densitometric analysis of RT-PCR products represented in (B). Results are expressed as the optical density of $\alpha 7$ -nAChR normalized by β -actin (n=5 experiments with 5 different animals). (D) RT-PCR analysis of nicotine stimulation of $\alpha 7$ -nAChR mRNA expression in ASMCs cultured for 48 hours in the absence or presence of indicated concentrations of nicotine. (E) Densitometric analysis of RT-PCR products represented in (D). Results are expressed as the optical density of $\alpha 7$ -nAChR normalized by β -actin as internal control (n=5 experiments with 5 different animals). (F) The effect of nicotine on $\alpha 7$ -nAChR mRNA expression as determined by real-time RT-PCR (n=3 experiments with 3 different animals). Total RNA was isolated from ASMCs cultured for 48 hours in the absence or presence of $10 \mu\text{M}$ nicotine. Results were expressed as n-fold difference in $\alpha 7$ -nAChR gene expression relative to β -actin gene. *Significant difference from untreated control condition ($P < 0.05$).

nAChRs or cytotoxicity. These results for the first time suggest that nicotine plays a role in Ca^{2+} homeostasis of ASMCs.

Expression of nAChR subtypes in rat ASMCs

To further examine the nAChR subtypes that maybe involved in the altered Ca^{2+} homeostasis, the expression of various nAChR subunits ($\alpha 1$ -7 and $\beta 1$ -4) in rat ASMCs was determined using RT-PCR. As shown in Fig. 3A, the expression of $\alpha 2$ -7, $\beta 2$, and $\beta 3$ nAChR

Fig. 4. Concentration and time-dependent effects of nicotine on $\alpha 7$ -nAChR protein expression in rat ASMCs. (A) Representative immunoblot showing $\alpha 7$ -nAChR protein expression in ASMCs cultured in the presence of nicotine at various concentrations for 48 hours. Anti- α -actin antibody was used as loading control. (B) Densitometric analysis of $\alpha 7$ -nAChR protein expression in ASMCs treated with nicotine at various concentrations. Results are expressed as the optical density of $\alpha 7$ -nAChR and normalized to α -actin levels (n=5 experiments with 3 animals). (C) Representative immunoblot showing $\alpha 7$ -nAChR protein expression in ASMCs harvested at different time-points after nicotine treatment. (D) Densitometric analysis of $\alpha 7$ -nAChR protein levels in ASMCs harvested at different time-points after nicotine treatment. Results are expressed as the optical density of $\alpha 7$ -nAChR and normalized to α -actin levels (n=4 experiments with 3 animals). *Significant difference from control untreated cells ($P < 0.05$).

RNA were clearly detected in rat ASMCs with $\alpha 7$ being the most abundant nAChR subtype followed by $\beta 2$, $\alpha 5$, $\alpha 6$, $\beta 3$, $\alpha 4$, $\alpha 3$, and $\alpha 2$. The transcripts of $\alpha 1$, $\beta 1$, and $\beta 4$ were not detected in ASMCs. Since $\alpha 7$ -nAChR is most abundantly expressed and possesses the unique property of the highest Ca^{2+} permeability among the nAChR subtypes [38-40], we focused our attention on the role of $\alpha 7$ -nAChR in nicotine-induced Ca^{2+} response.

Nicotine increased $\alpha 7$ -nAChR gene expression in ASMCs

We evaluated the effect of nicotine exposure on $\alpha 7$ -nAChR expression in the cultured ASMCs. RT-PCR analysis revealed a time-dependent nicotine (10 μ M) induction of $\alpha 7$ -nAChR mRNA, with a maximal increase after 48 hours of culture (Fig. 3B and C). The effect of nicotine on $\alpha 7$ -nAChR expression was concentration-dependent (10^{-8} to 10^{-4} M); the mRNA level was approximately doubled at 10 and 100 μ M nicotine (Fig. 3D and E). Further increase in nicotine concentration reduced the increase in $\alpha 7$ -nAChR expression in ASMCs (data not shown). The observation of nicotine-induced $\alpha 7$ -nAChR upregulation was confirmed by quantitative real-time RT-PCR (Fig. 3E), which showed nicotine treatment at 10 μ M for 48 hours caused a 1.7-fold increase in $\alpha 7$ -nAChR mRNA expression compared to the control. Consistent with the changes observed in $\alpha 7$ -nAChR mRNA, $\alpha 7$ -nAChR protein level was increased in nicotine treated cells in a concentration-dependent fashion, with 2-fold increase at 10^{-5} M compared to the control (Fig. 4A and 5B). The stimulatory effect of nicotine on $\alpha 7$ -nAChR protein expression in ASMCs was maximal at 48 hours of treatment (Fig. 4C and 4D). These results clearly suggest that nicotine can auto-regulate the expression of its own receptors in ASMCs

Effect of acute application of nicotine on $[Ca^{2+}]_i$ in control ASMCs and chronic nicotine treated ASMCs

In addition to the chronic effect of nicotine exposure on resting $[Ca^{2+}]_i$, acute exposure of nicotine evoked a rapid increase in $[Ca^{2+}]_i$ in ASMCs. Nicotine-induced Ca^{2+} transient is concentration-dependent (Fig. 5A and B). At a lower concentration of 10^{-5} M, nicotine caused a mild sustained Ca^{2+} increase with an average $\Delta[Ca^{2+}]_i$ of 50.6 ± 5.1 nM, whereas 10^{-4} M nicotine evoked a robust biphasic Ca^{2+} response with a peak $\Delta[Ca^{2+}]_i$ of 330.4 ± 28.5 nM. The

Fig. 5. Acute nicotine-induced Ca^{2+} response in control and nicotine-pretreated ASMCs. (A) Mean trace showing time-course of the change in $[Ca^{2+}]_i$ evoked by the application of 10, 50, and 100 μM nicotine. (B) Mean peak change in $[Ca^{2+}]_i$ induced by 10, 50, and 100 μM nicotine generated from 78 cells in 4 experiments. * indicates significant

difference ($P < 0.05$) from 10 μM , and # indicates significant difference between 50 μM and 100 μM . (C) Ca^{2+} response induced by 50 μM nicotine in a control cell (left panel) and a cell after 48-hour incubation with 1 μM nicotine (right panel). (D) Summary data quantifying the mean peak Ca^{2+} response to nicotine with or without chronic exposure to nicotine. Mean response was taken from 93 cells ($n = 4$ experiments). *Significant difference from control, $P < 0.05$.

nicotine-induced Ca^{2+} response was further examined in cultured ASMCs with or without pretreatment with 1 μM nicotine for 48 hours. Acute nicotine-induced Ca^{2+} transients were significantly enhanced in ASMCs after 48 hours of nicotine treatment as compared to control ASMCs (Fig. 5C and D). The peak $\Delta[Ca^{2+}]_i$ induced by 50 μM nicotine was 320.3 ± 31.1 nM in nicotine-pretreated ASMCs and 191.2 ± 23.4 nM in control ASMCs. There was no significant difference between the sustained Ca^{2+} response of the nicotine-treated (145.2 ± 13.9 nM) and the control (121.7 ± 14.1 nM, $P > 0.05$) ASMCs. These results suggest that chronic exposure to a low level of nicotine enhances the nAChR mediated peak Ca^{2+} response, consistent with the upregulation of $\alpha 7$ -nAChR expression in the nicotine-treated ASMCs.

Effects of nAChR antagonists and $\alpha 7$ -nAChR siRNA on nicotine-induced Ca^{2+} response in ASMCs

The role of $\alpha 7$ -nAChR in the nicotine-induced Ca^{2+} response was further examined pharmacologically in ASMCs, 100 μM nicotine elicited a biphasic response with a peak transient response of 293.1 ± 31.1 nM followed by a sustained phase of 142.3 ± 15.6 nM ($n = 4$, measured at 500-700 s after nicotine application) in control ASMCs. MLA (20 nM), a competitive antagonist specific for $\alpha 7$ -nAChR, reversibly inhibited nicotine-induced Ca^{2+} response by $62.1 \pm 2.2\%$ ($n = 5$, $P < 0.05$) in the peak and $50.2 \pm 1.5\%$ ($P < 0.05$) in the sustained Ca^{2+} response (Fig. 6A,B). Another specific nAChR antagonist α -bungarotoxin (10 μM) also significantly inhibited the nicotine-induced Ca^{2+} response (Fig. 6C,D). The peak Ca^{2+} responses were 220.3 ± 15.4 nM ($n = 5$) and 96.5 ± 11.2 nM ($n = 5$, $P < 0.05$), and the sustained Ca^{2+} responses were 113.5 ± 15.6 nM ($n = 5$) and 61.8 ± 9.6 nM ($n = 5$, $P < 0.05$) in the absence and presence of α -bungarotoxin, respectively. To further examine if $\alpha 7$ -nAChR indeed mediates nicotine-induced Ca^{2+} response in ASMCs, ASMCs were transfected with $\alpha 7$ -nAChR specific

Fig. 6. Effects of $\alpha 7$ -nAChR antagonists on nicotine-induced Ca^{2+} response in ASMCs. (A) Ca^{2+} response induced by 100 μM nicotine in the absence (left panel) and presence (right panel) of the $\alpha 7$ -nAChR antagonist, methyllycaconitine (MLA) (20 nM). (B) Average changes in $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i$) in the peak and the sustained

nicotine-induced Ca^{2+} response in the absence and presence of MLA. Average data were taken from 78 cells ($n = 4$ experiments). (C) Ca^{2+} response induced by 100 μM nicotine in the absence (left panel) and the presence (right panel) of the $\alpha 7$ -nAChR antagonist, alpha-bungarotoxin (α -BuTX) (10 μM). (D) Average $\Delta[Ca^{2+}]_i$ in the peak and sustained nicotine-induced Ca^{2+} response in the presence or absence of α -BuTX. Mean response was taken from 108 cells ($n = 5$ experiments). * indicates significant difference from control, $P < 0.05$.

Fig. 7. (A) Immunoblot showing the expression of $\alpha 7$ -nAChR protein in control ASMCs and in ASMCs transfected with siRNA specifically against $\alpha 7$ -nAChR or a control sequence. (B) Mean data showing % inhibition of $\alpha 7$ -nAChR protein expression in ASMCs transfected with $\alpha 7$ -nAChR siRNA ($n = 3$ experiments). (C) Ca^{2+} response induced by 100 μM nicotine in ASMCs transfected with $\alpha 7$ -nAChR siRNA and a control sequence. (D) Summary data quantifying the mean peak $\Delta[Ca^{2+}]_i$ in response to nicotine in cells with and without transfected with $\alpha 7$ -nAChR specific SiRNA. Mean response was taken from 68 cells ($n = 4$ experiments). * indicates significant difference from control, $P < 0.05$.

siRNA and scrambled control. siRNA transfection specifically decreased $\alpha 7$ -nAChR protein expression after 72 hours by $65.4 \pm 1.8\%$ ($n = 3$), whereas negative control sequence did not substantially change the expression of the protein (Fig. 7A and B) Furthermore, nicotine-induced Ca^{2+} response was significantly abrogated in ASMCs transfected with $\alpha 7$ -nAChR

specific siRNA (Fig. 7C). The peak Ca^{2+} responses were 250.2 ± 23.4 nM ($n = 4$) and 61.3 ± 8.2 nM ($n = 4$, $P < 0.05$) in ASMCs transfected with $\alpha 7$ -nAChR specific siRNA and scrambled sequence, respectively (Fig. 7D), while the sustained Ca^{2+} responses were unaffected (control: 102.2 ± 11.6 nM; siRNA: 98.2 ± 10.4 nM). These results clearly suggest that $\alpha 7$ -nAChR is responsible for a major component of the nicotine-induced Ca^{2+} response in ASMCs.

Discussion

Chronic obstructive pulmonary disease (COPD) is an inflammatory airway disease characterized by different patterns of airway remodeling [41]. The decrease in lung function in COPD is associated with an increase in bronchial smooth muscle [42], which is likely the most important abnormality responsible for the airway narrowing in response to bronchoconstricting stimuli [43]. The mechanisms underlying such remodeling of smooth muscle remain largely unclear; and the role of nicotine in this process has not been explored. In this study, we found that chronic nicotine treatment of ASMCs causes an increase in baseline $[Ca^{2+}]_i$, which is an important determinant of airway smooth muscle growth and proliferation [44], in a concentration- and time-dependent fashion. We further found that rat ASMCs express multiple nAChR subtypes, including $\alpha 2$ - $\alpha 7$, as well as $\beta 2$ and $\beta 3$. This is consistent with a previous report using immunohistochemistry showing that $\alpha 4$ and $\alpha 7$ -nAChR proteins are expressed in mouse tracheal smooth muscle [45]. Among the nAChRs in ASMCs, $\alpha 7$ -nAChR is one of the most abundant subtypes and possesses the unique functional property of high Ca^{2+} permeability [38-40]. Therefore, one may speculate that nicotine stimulates Ca^{2+} influx via $\alpha 7$ -nAChR and perhaps other nAChRs to activate Ca^{2+} -dependent pathways including CaM kinase II [13, 46] to play a contributing role in the proliferation and contraction of ASMCs.

The pathological effect of nicotine on ASMC functions is supported by the observation that nicotine exposure increased $\alpha 7$ -nAChR expression in ASMCs. This is consistent with previous studies showing that chronic exposure to nicotine up-regulate the expression of nAChRs, including $\alpha 7$ -nAChR [47-49], in neuronal cells. The increase in $\alpha 7$ mRNA level in ASMCs suggests that nicotine, by binding to its receptors, enhances $\alpha 7$ -nAChR transcription. Similar to our observation, chronic nicotine exposure causes the increase in mRNA levels of several nAChR subtypes in rat bladder [50]. In neurons, some nAChR subtypes undergo up-regulation, changes in stoichiometry and increase in active-state (functional up-regulation) after chronic nicotine exposure [48]. Up-regulation of $\alpha 7$ -nAChRs at the post-transcriptional levels has also been reported in neuronal cell cultures [49]. The transcriptional and post-transcriptional regulation of nAChRs by nicotine could be related to differences between neurons and smooth muscle cells. A recent study suggest that nicotine-induced alterations of native neuronal nicotinic receptors are caused by multiple mechanisms [51], and the mechanisms could vary for different nAChR subtypes [52]. Hence, the regulatory mechanisms responsible for nicotine-induced nAChR expression in ASMCs require further investigations.

The present study also demonstrated for the first time that acute application of nicotine activates concentration-dependent Ca^{2+} response in ASMCs, suggesting that the nAChRs expressed are functional Ca^{2+} pathways in ASMCs. Moreover, chronic nicotine exposure increases the nicotine-induced peak Ca^{2+} response in ASMCs. This clearly suggests that nicotine induced persistent changes in nAChR functions, leading to an increased nAChR dependent activity in ASMCs. This is consistent with the increased expression of $\alpha 7$ -nAChR mRNA and protein, and the upregulation is associated with increased functional nAChR channels in ASMCs [53]. The increase in the nicotine-induced Ca^{2+} response in the nicotine-treated cells could also be mediated in part by increased trafficking and membrane insertion of functional nAChRs, which is well-documented in neuronal cells [54]. However, whether chronic nicotine exposure also increases the conductance and activity of individual nAChR in ASMCs cannot be determined without single-channel recording on ASMCs.

The fact that nicotine is capable of inducing acute increase in $[Ca^{2+}]_i$ of ASMCs led us to further examine the pathways involved using specific nAChR blockers. Although mechanisms

other than nAChR activation cannot be ruled out in the nicotine-induced increase in cytosolic Ca^{2+} , the effective inhibition of the Ca^{2+} response by the $\alpha 7$ -nAChR antagonists MLA and α -bungarotoxin indicates that $\alpha 7$ -nAChR activation plays a major role in this process. The role of $\alpha 7$ -nAChR in nicotine-mediated Ca^{2+} signaling was further confirmed in the RNA interference experiments, where ASMCs transfected with siRNA that selectively suppresses the expression of $\alpha 7$ -nAChR substantially attenuated the nicotine-induced transient Ca^{2+} response. These results provide strong evidence that $\alpha 7$ -nAChR is a major Ca^{2+} pathway mediating the nicotine-induced Ca^{2+} responses in ASMCs cells.

It has to be mentioned, however, that our study is focused mainly on the $\alpha 7$ -nAChR because of its abundance in ASMCs and its Ca^{2+} permeability. Our results, however, do not exclude the possible participation of other nAChR subtypes in the nicotine-induced Ca^{2+} response and alterations in Ca^{2+} homeostasis in ASMCs. In fact, the partial inhibition of the sustained phase of nicotine-induced Ca^{2+} response by the $\alpha 7$ -nAChR specific antagonist MLA and the persistent appearance of the sustained component in the siRNA transfected cells suggest that other nAChR subtypes expressed in ASMCs are involved particularly in the sustained Ca^{2+} response. The molecular identity of nAChR subtypes responsible for the sustained nicotinic Ca^{2+} response, which can be relevant for ASMCs proliferation, warrant further future investigations.

In conclusion, the present study demonstrates that (1) multiple nAChR are expressed in ASMCs; (2) chronic exposure of ASMCs increases resting $[Ca^{2+}]_i$ in ASMCs; (3) the expression of $\alpha 7$ -nAChR is enhanced in ASMCs after chronic nicotine exposure; (4) acute application of nicotine elicits Ca^{2+} response in ASMCs and the Ca^{2+} response is enhanced in ASMCs chronically exposed to nicotine; and (5) the acute nicotine-induced Ca^{2+} response is mediated in part via $\alpha 7$ -nAChR. These results highlight an important role of $\alpha 7$ -nAChR in ASMC and suggest an intriguing mechanism that chronic nicotine exposure may activate a feed-forward mechanism to enhance $\alpha 7$ -nAChR dependent effects in ASM contractility and remodeling. Future studies focusing on the relative roles of $\alpha 7$ -nAChR and other nAChR subtypes will likely generate further interesting findings in nicotine-induced ASMC proliferation and airway remodeling.

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