Calcium transient evoked by nicotine in isolated rat vagal pulmonary sensory neurons

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Xu J, Yang W, Zhang G, Gu Q, Lee L-Y. Calcium transient evoked by nicotine in isolated rat vagal pulmonary sensory neurons. Am J Physiol Lung Cell Mol Physiol 292: L54–L61, 2007. First published August 18, 2006; doi:10.1152/ajplung.00182.2006.—It has been shown that inhaled cigarette smoke activates vagal pulmonary C fibers and rapidly adapting receptors (RARs) in the airways and that nicotine contained in the smoke is primarily responsible. This study was carried out to determine whether nicotine alone can activate pulmonary sensory neurons isolated from rat vagal ganglia; the response of these neurons was determined by fura-2-based ratiometric imaging. The results showed: 1) Nicotine (10−4 M, 20 s) evoked a transient increase in intracellular Ca2+ concentration ([Ca2+]i) in 175 of the 522 neurons tested ([Δ[Ca2+]i] = 142.2 ± 12.3 nM); the response was reproducible, with a small reduction in peak amplitude in the same neurons when the challenge was repeated 20 min later. 2) A majority (59.7%) of these nicotine-sensitive neurons were also activated by capsaicin (10−7 M). 3) 1,1-Dimethyl-4-phenylpiperazinium iodide (DMPP; 10−4 M, 20 s), a selective agonist of the neuronal nicotinic acetylcholine receptors (NnAChRs), evoked a pattern of response similar to that of nicotine. 4) The responses to nicotine and DMPP were either totally abrogated or markedly attenuated by hexamethonium (10−4 M). 5) In anesthetized rats, right atrial bolus injection of nicotine (75–200 μg/kg) evoked an immediate (latency <1–2 s) and intense burst of discharge in 47.8% of the pulmonary C-fiber endings and 28.6% of the RARs tested. In conclusion, nicotine exerts a direct stimulatory effect on vagal pulmonary sensory nerves, and the effect is probably mediated through an activation of the NnAChRs expressed on the membrane of these neurons.

lungs; C fibers; airway irritation; cigarette smoke; 1,1-dimethyl-4-phenylpiperazinium iodide

CIGARETTE SMOKE CAUSES airway irritation and coughing and is undoubtedly one of the most common inhaled irritants to the human respiratory tract. It was previously demonstrated in our laboratory (13, 16, 19) that inhalation of one or a few breaths of cigarette smoke can stimulate both vagal bronchopulmonary C-fiber afferents and rapidly adapting receptors (RARs) in the lungs in various animal species. More importantly, we have shown that this stimulatory effect is primarily caused by nicotine contained in the cigarette smoke (16, 19). Our follow-up studies (18) conducted in healthy nonsmokers have further confirmed that the irritant effect of inhaled cigarette smoke in the human respiratory tract is indeed generated by the action of nicotine. However, nicotinic acetylcholine receptors are present on a variety of cells (e.g., cholinergic ganglion neurons, smooth muscles) in the airways, and it is also known that nicotine can induce bronchoconstriction (14, 20). Thus whether the stimulatory effect was due to a direct action of nicotine on these nerve endings or secondary to a nicotine-induced bronchoconstrictive effect could not be determined in these studies; the latter possibility was supported by the observation that the delayed stimulatory effect of cigarette smoke on RARs was attenuated when bronchoconstriction was prevented by pretreatment with a bronchodilator (16).

Our hypothesis is that nicotine can stimulate these afferents directly by activation of neuronal nicotinic acetylcholine receptors (NnAChRs) expressed on the membrane of these sensory neurons. To test this hypothesis, the present study was carried out in isolated pulmonary sensory neurons from adult Sprague-Dawley rats. One well-documented, characteristic response evoked by activation of NnAChRs is an increase in intracellular Ca2+ concentration ([Ca2+]i) in the neuron by direct passage of calcium through the receptor channel (4, 26). Therefore, we studied the response of isolated pulmonary sensory neurons to nicotine by quantitative measurements of the intracellular Ca2+ transient in this study. To further test the effect of nicotine on the sensory terminals of these neurons, we studied the electrophysiological responses of single-unit pulmonary vagal afferents to right atrial injection of nicotine in anesthetized rats.

MATERIALS AND METHODS

The procedures described below were performed in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. These animal experimental protocols were also approved by the University of Kentucky Institutional Animal Care and Use Committee.

In Vitro Study

Labeling vagal pulmonary sensory neurons with Dil. Cell bodies of vagal sensory nerves arising from airways and lungs reside in nodose and intracranial jugular ganglia. These sensory neurons were identified by retrograde labeling from the lungs with the fluorescent tracer Dil (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate), as described previously (17). Briefly, young adult Sprague-Dawley rats (~160 g) were anesthetized with an intraperitoneal injection of pentobarbital sodium (40 mg/kg) and intubated with a polyethylene catheter (PE-150) with its tip positioned in the trachea above the thoracic inlet. Dil was initially sonicated and dissolved in ethanol, diluted in saline [1% (vol/vol) ethanol] and then instilled into the lungs (0.2 mg/ml; 0.2 ml × 2) with the animal’s head tilted upward at ~30°.

Isolation and culture of nodose and jugular ganglion neurons. After 7–10 days, an interval previously determined to be sufficient for Dil to diffuse to the cell body, the rats (170–260 g) were anesthetized with halothane inhalation and decapitated. The head was immediately frozen, sectioned into 300-μm-thick slices, and the sections were transferred to a 37°C water bath. After 15 min in the water bath, the slices were transferred to a 37°C perfusion chamber filled with an oxygenated-submerged solution consisting of (in mM): 125 NaCl, 2.5 KCl, 2 CaCl2, 1.25 MgCl2, 10 dextrose, and 5 Hepes (pH 7.4). After an additional 15 min in the chamber, the slices were transferred to a holding chamber filled with the same solution but with 2 μM tetrodotoxin (TTX). After 1 h in the holding chamber, the slices were transferred to a 37°C extracellular recording chamber filled with the same solution as in the holding chamber but with 2 μM TTX and 0.2 μM nigericin. The extracellular solution was oxygenated with 95% O2 and 5% CO2, and the slices were allowed to equilibrate for 15–20 min in the recording chamber. After equilibration, the slices were visually inspected to ensure that the isolated vagal pulmonary afferents had not been damaged during slicing or perfusion. Isolated vagal pulmonary afferents that appeared to be intact were selected for recording.

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immersed in ice-cold Hanks’ balanced salt solution. Nodose and jugular ganglia were extracted under a dissecting microscope and placed in ice-cold Dulbecco’s modified Eagle’s medium-F-12 (DMEM-F-12) 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 g, 5 min), and the supernatant was aspirated. The cell pellet was resuspended in 0.05% trypsin in Hanks’ balanced salt solution for 5 min and centrifuged (150 g, 5 min); the pellet was then resuspended in a modified DMEM-F-12 solution [DMEM-F-12 supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, and 100 µM MEM nonessential amino acids] and gently triturated with a small-bore fire-polished Pasteur pipette. The dispersed cell suspension was centrifuged (500 g, 8 min) through a layer of 15% bovine serum albumin to separate the cells from the myelin debris. The pellets were resuspended in the modified DMEM-F-12 solution supplemented with 50 ng/ml 2.5s nerve growth factor, plated onto poly-L-lysine-coated glass coverslips, and then incubated overnight (5% CO2 in air at 37°C). 

Intracellular Ca2+ measurement. Intracellular Ca2+ was monitored with the fluorescent Ca2+ indicator fura-2 AM as described previously (11). Briefly, cells were loaded with 5 µM fura-2 AM for 30 min at 37°C, then rinsed (3x) with standard extracellular solution (ECS; in mM: 136 NaCl, 5.4 KCl, 1.8 CaCl2, 1 MgCl2, 33 NaH2PO4, 10 glucose, 10 HEPES, pH 7.4), and allowed to deesterify for at least 30 min before use. Ratiometric Ca2+ imaging was performed with a Zeiss fluorescent inverted microscope equipped with a variable filter wheel (Sutter Instruments; Novato, CA) and digital charge-coupled device camera (Princeton Instruments; Trenton, NJ). Dual images (340- and 380-nm excitation, 510-nm emission) were collected, and pseudocolor ratiometric images were monitored during the experiments with Axon Imaging Workbench software (Axon Instruments; Union City, CA). The imaging system was standardized with a two-point calibration, using a Ca2+-free standard (−) and a Ca2+-saturated standard (+). Both standards contained 11 µM fura-2 [44 µl of 10 mM fura-2 penta-K+ salt, 8 ml of 20 mM HEPES-Na (pH 7.4), 32 ml H2O] and were prepared as follows: − standard: 18 ml fura-2, 1.98 ml of 10 mM EGTA-Na (pH 7.6); + standard: 18 ml fura-2, 1.98 ml of 10 mM CaCl2. The parameters used for the two-point calibration include the dissociation constant of fura-2 (Kd; 225), the ratio values for the − and + concentration standards (Rmin and Rmax), and the fluorescence intensities at 380-nm excitation for the − and + concentration standards (Demin and Demax). [Ca2+]i (in nM) was calculated according to the following equation (10): [Ca2+]i = Kd(R − Rmin)/(Rmax − R)(Demin/Demax), where R represented fluorescence intensity ratio between 340 and 380 nm. Typical Rmin, Rmax, and Demin/Demax values were 0.59, 2.54, and 2.27, respectively.

Experimental protocols. After the incubation period with fura-2 AM, the coverslip containing cells was mounted into a chamber (0.2 ml) and then flushed with 0.2 ml/min; a complete change of the ECS occurred in 6 s. At the onset of each test, the valve was switched by the computer command signal, and the perfusate was changed to the ECS containing the chemical stimulant (e.g., nicotine) prepared in the desired concentration and delivered via the manifold of the same perfusion system for 20 s. Four study series were carried out to determine 1) whether nicotine generated an excitatory effect on isolated pulmonary nodose and jugular ganglion neurons and, if so, whether the effect of nicotine was concentration dependent; 2) whether 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP), an agonist of NnAchRs, also evoked a stimulatory effect on these neurons and whether DMPP and nicotine activated the same neurons in a similar pattern; 3) the effect of hexamethonium, an antagonist of nicotinic acetylcholine receptors, on the responses of these neurons to nicotine and DMPP; and 4) the distribution of nicotine-sensitive pulmonary sensory neurons. The sequence of chemical challenges was alternated between neurons to achieve a balanced design. To avoid desensitization of the neurons to the chemical stimulants, each coverslip was used for only one study series and 15–20 min elapsed between two chemical applications. KCl solution (final concentration 60 mM) was perfused at the end of each experimental run to test for cell viability. The peak amplitude of the Ca2+ transient (Δ[Ca2+]i) evoked by a chemical stimulant was measured as the difference between the 6-s average at peak and the 30-s average at baseline.

In Vivo Study: Recording of Single-Fiber Activity of Pulmonary Afferents

To determine whether nicotine can also activate sensory terminals located in the lungs, afferent activities of pulmonary C fibers, RARs, and slowly adapting receptors (SARs), were recorded in anesthetized rats as described in detail previously (14). Briefly, Sprague-Dawley rats (330–470 g) were initially anesthetized with an intraperitoneal injection of α-chloralose (100 mg/kg; Sigma, St. Louis, MO) and urethane (500 mg/kg; Sigma) dissolved in a borax solution (2%; Sigma); supplemental doses of these anesthetics were injected intravenously to maintain abolition of pain reflexes. A short tracheal cannula was inserted after a tracheostomy, and tracheal pressure (P0) was measured (Validyne MP 45–28, Northridge, CA) via a side port of the tracheal cannula. Chest was opened by a midline thoracotomy for later identification of the locations of sensory endings in the lungs. The expiratory outlet of the respirator was placed under 3-cmH2O pressure to maintain a near-normal functional residual capacity. Volume and frequency of the respirator were set at 8–10 ml/kg and 50 breaths/min, respectively. One femoral artery was cannulated for recording the arterial blood pressure (ABP). For right atrial injection of pharmacological agents, the left jugular vein was cannulated and a catheter was advanced until its tip was positioned just above the right atrium. The volume of each bolus injection was 0.15 ml, which was first injected into the catheter (dead space ~0.2 ml) and then flushed into the circulation by an injection of 0.4 ml of saline. Body temperature was maintained at ~36°C by means of a heating pad placed under the animal. The right cervical vagus nerve was sectioned as rostrally as possible, and the caudal end of the cut vagus was placed on a small dissecting platform and immersed in a pool of mineral oil. A thin filament was teased away from the desheathed nerve trunk and placed on a miniature platinum-iridium electrode. Action potentials were amplified (Grass P511K, West Warwick, RI), monitored by an audio monitor (Grass AM8RS), and displayed on an oscilloscope (Tektronix 2211, Beaverton, OR). The thin filament was further split until the action potentials arising from a single unit were electrically isolated. Our criteria for identifying these different types of lung afferents were described in detail previously (13). The signals of the fiber activity (FA), P0, and ABP were analyzed with an online data acquisition system (Biocybernetics TS-100, Taipei, Taiwan) in 0.5-s intervals.

Chemicals

DMEM-F-12, trypsin, and nerve growth factor were obtained from Invitrogen (Carlsbad, CA). Fura-2 AM and DiI were purchased from Molecular Probes (Eugene, OR). Nicotine, DMPP, capsaicin, and hexamethonium were obtained from Sigma. In the study of isolated neurons, a stock solution of capsaicin (10−3 M) was prepared in a vehicle of 10% Tween 80, 10% ethanol, and 80% ECS and then diluted to 10−7 M before each experiment; all other pharmacological agents were prepared daily in ECS. In the in vivo study, the desired concentrations of the pharmacological agents were prepared in a similar manner, except that isotonic saline, instead of ECS, was used as vehicle.
**Statistical Analysis**

Data were analyzed by a one-way or two-way analysis of variance (ANOVA). When the ANOVA showed a significant interaction, pairwise comparisons were made with a post hoc analysis (Fisher’s least significant difference). Results were considered significant when $P < 0.05$. Data are means ± SE.

**RESULTS**

**In Vitro Study**

A total of 522 isolated nodose and jugular pulmonary neurons was studied in 25 rats. The average resting $[\text{Ca}^{2+}]_{i}$ was 55.3 ± 1.5 nM. Only neurons that responded to KCl challenge (60 mM, 15 s) with a rapid and reversible increase in $[\text{Ca}^{2+}]_{i}$.
abrupt and transient increase in \([Ca^{2+}]_i\). Application of nicotine for 20 s evoked an 
sensory neurons. A pattern in 2 different nodose neurons (diameter 20.3 
neurons: experimental records illustrating that the same concentrations (3 
either nicotine or DMPP. No noticeable difference was found between their responses to 
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Nicotine evoked \(Ca^{2+}\) transient in rat vagal pulmonary sensory neurons. Application of nicotine for 20 s evoked an 
abrupt and transient increase in \([Ca^{2+}]_i\), in a concentration-

Dependent manner (Fig. 1, A and B). A low concentration (10\(^{-5}\) M) of nicotine caused only a very mild \(Ca^{2+}\) transient 
with an average \(\Delta[Ca^{2+}]_i\) of 26.7 \pm 14.3 nM, whereas 10\(^{-4}\) M nicotine evoked a significantly higher \(\Delta[Ca^{2+}]_i\) (142.2 \pm 15.1 
nM; \(P < 0.05, n = 25\)). The \(Ca^{2+}\) transient evoked by this high 
dose of nicotine (10\(^{-4}\) M, 20 s) was reproducible in the same sensory neurons after washout for 20 min (see, e.g., Fig. 1C); 
the response evoked by the second nicotine challenge (125.8 \pm 19.4 nM) was slightly but significantly smaller compared with that 
generated by the first challenge (142.3 \pm 21.1 nM) (\(P < 0.05, n = 18\); Fig. 1D).

DMPP mimicked the stimulatory effect of nicotine in rat 
vagal pulmonary sensory neurons. DMPP, a relatively specific 
agonist of NnAChRs, also activated these pulmonary sensory 
neurons in a concentration-dependent manner (Fig. 2, A and B). 
The response to repeated application of a high dose of DMPP 
(10\(^{-4}\) M, 20 s) also showed a small but significant decrease in \(Ca^{2+}\) transient (1st DMPP: 122.6 \pm 24.2 nM, 2nd DMPP: 
101.8 \pm 21.5 nM; \(P < 0.05, n = 12\)) (Fig. 2, C and D).

The \(Ca^{2+}\) transients evoked by the same molar concentration (3 \times 10\(^{-5}\) M, 20 s) of nicotine and DMPP exhibited a very 
similar pattern in the same pulmonary sensory neurons (see, e.g., Fig. 3). At this particular concentration, the average 
amplitude of \(Ca^{2+}\) transient evoked by DMPP was slightly 
higher in the same cells (nicotine 66.6 \pm 22.9 nM, DMPP 
85.3 \pm 23.4 nM; \(P < 0.05, n = 12\); the sequence of nicotine 
and DMPP challenges was reversed in about half of these 
neurons to avoid the influence of desensitization.

Effects of nicotine and DMPP were prevented by hexamethonium in pulmonary sensory neurons. Pretreatment with 
hexamethonium (10\(^{-4}\) M, 5 min), an antagonist of nicotinic 
acetylcholine receptors, almost completely blocked the \(Ca^{2+}\) transient evoked by the same concentration of nicotine (10\(^{-4}\) 
M, 20 s) (see, e.g., Fig. 4A), and this blocking effect was 
largely reversed after 15–20 min washout (control 158.5 \pm 
44.6 nM, after hexamethonium 13.3 \pm 8.2 nM; \(P < 0.05, n = 17\) (Fig. 4B). Similarly, the stimulatory

Fig. 3. Nicotine- and DMPP-evoked \(Ca^{2+}\) transients in the same vagal sensory neurons: experimental records illustrating that the same concentrations (3 \times 10\(^{-5}\) M, 20 s) of nicotine and DMPP evoked \(Ca^{2+}\) transients in a similar pattern in 2 different nodose neurons (diameter 20.3 \(\mu\)m in A and 31.2 \(\mu\)m in B). Note that the neuron in B but not in A was sensitive to 10\(^{-7}\) M capsaicin, whereas KCl (6 \times 10\(^{-5}\) M, 15 s), which was applied to test cell viability at the end of the experiment, immediately evoked an intense but reversible \(Ca^{2+}\) transient in both neurons.

Fig. 4. Nicotine- and DMPP-evoked \(Ca^{2+}\) transients were prevented by hexamethonium in isolated rat vagal pulmonary sensory neurons. A and C: experimental records illustrating that pretreatment with hexamethonium (Hex; 10\(^{-5}\) M, 5 min) almost completely blocked the \(Ca^{2+}\) transient evoked by the same concentration of nicotine (10\(^{-4}\) M, 20 s) (see, e.g., Fig. 4A), and this blocking effect was largely reversed after 15–20 min washout (control 158.5 \pm 
44.6 nM, after hexamethonium 13.3 \pm 4.7 nM, after washout 
106.7 \pm 30.9 nM; \(n = 17\) (Fig. 4B). Similarly, the stimulatory
sensitivity in these neurons. We should also point out that whether the size of cultured neurons accurately represents that of the cell body before these neurons were removed from intact animals is not known.

In Vivo Study: Response of Pulmonary Afferents to Nicotine Injection

To determine the effect of nicotine on the sensory terminals of these pulmonary neurons, a total of 37 single-unit pulmonary afferents (23 pulmonary C fibers, 7 RARs, and 7 SARs) was studied in 11 rats. Right atrial bolus injection of nicotine (75–200 μg/kg) triggered an intense burst of action potentials immediately (within 1–2 s) after the injection in 11 of the 23 C fibers tested (see, e.g., Fig. 6); after injection of nicotine, the difference between the peak FA (2-s average) and the baseline FA (5-s average) (ΔFA) was 9.6 ± 2.7 impulses (imp)/s (n = 23; P < 0.01). Similarly, injection of DMPP stimulated 10 of the 23 C fibers (ΔFA = 11.7 ± 2.7 imp/s; n = 23, P < 0.01). There was no significant difference between the peak responses to nicotine and DMPP in the same C fibers (n = 23, P > 0.05). In contrast, only two of the seven RARs and none of the seven SARs were activated immediately after the nicotine and DMPP injection. Coincidently, one of the two nicotine-sensitive RARs also exhibited a mild sensitivity to capsaicin (Fig. 6). All seven RARs had low or no baseline activity, and four of them were also activated by lung deflation (see, e.g., Fig. 6). Overall, a higher percentage (47.8%) of C-fiber afferents than of RARs (28.6%) exhibits nicotine sensitivity.

Bolus injection of nicotine evoked an initial bradycardia, probably resulting from the C-fiber stimulation, and a delayed and longer-lasting increase in ABP, presumably by activating the sympathetic nervous system (16).

DISCUSSION

The results of this study clearly show that nicotine evoked a rapid and transient increase in [Ca\(^{2+}\)], in 33.5% of the isolated rat vagal pulmonary sensory neurons. In the anesthetized rat preparation, nicotine injected into the right atrium also triggered an abrupt burst of discharge in 47.8% of the pulmonary C-fiber endings and 28.6% of the RARs. The nicotine-evoked Ca\(^{2+}\) transient was concentration dependent and almost completely abrogated by a pretreatment with hexamethonium, suggesting the involvement of nicotinic acetylcholine receptors in this action. The fact that DMPP, a selective agonist of NnAChRs, evoked a similar pattern of response in the same neurons lends additional support to this conclusion.

Activation of NnACHRs either by nicotine or DMPP consistently triggers an abrupt rise in [Ca\(^{2+}\)] in these neurons, and the response is readily reversible. It is well documented that intracellular Ca\(^{2+}\) is an important signal transduction molecule in neurons and plays a critical role in the regulation of membrane excitability, neurotransmitter release, synaptic transmission, and other important biological functions. In this study, the responses to repeated challenges of either nicotine or DMPP were slightly but consistently reduced (Figs. 1 and 2), especially if insufficient time (<10 min) was allowed for recovery. Desensitization is a well-recognized phenomenon for the entire nicotinic receptor family, including NnACHRs, that represents a classic form of allosteric protein behavior (4, 25). Desensitization of NnACHRs is a subunit-dependent property that
plays an important part in curtailing excessive receptor stimulation and preventing excitotoxicity resulting from Ca\(^{2+}\) influx via the activation of NnAChRs. During chronic exposure to nicotine, desensitization may also lead to receptor upregulation (5), and its potential role in the regulation of NnAChR function in pulmonary sensory nerves of chronic smokers has not been explored.

NnAChRs are a heterogeneous family of pentameric ligand-gated ion channels that have been localized in the peripheral nervous system as well as in the central nervous system. Approximately 50% of the sensory neurons cultured from trigeminal and dorsal root ganglia express functional NnAChRs (6, 8, 22). Nine \(\alpha (\alpha_2-\alpha_{10})\)- and three \(\beta (\beta_2-\beta_3)\)-subunits have been identified by molecular cloning (9), and additional new NnAChR subunits may still be identified. These subunits assemble in a number of different combinations to form various subtypes of NnAChRs (24). Both agonist and antagonist rank-order profiles of these NnAChRs are subunit dependent. For example, both \(\alpha_2\beta_2\)- and \(\alpha_2\beta_3\)-subunits are expressed in rat trigeminal neurons; the agonist potency is nicotine > DMPP > ACh in the former, whereas it is DMPP > ACh > nicotine in the latter (24). We cannot compare the potency between nicotine and DMPP in these pulmonary sensory neurons because complete concentration-response curves were not established in our study. The distribution of these different subunits in different types of pulmonary afferent nerves (pulmonary C fibers vs. RARs) is not known (27), and their role in regulating the electrophysiological and chemosensitive properties of these neurons remains to be determined.

The majority of afferent activities arising from endings located in the airways and lung are conducted in vagus nerves and their branches. Cell bodies of these afferent fibers reside in nodose and intracranial jugular ganglia. These vagal afferents can be generally classified into three major types, based on the standard criteria (3, 13, 21): SARs (or stretch receptors), RARs (or irritant receptors), and bronchopulmonary C fibers. Afferent discharges from the first two types of receptors are conducted by large-diameter, high-conduction-velocity, myelinated (A) fibers, whereas the last type is conducted by small-diameter, low-conduction-velocity, nonmyelinated (C) fibers (3, 13, 21). Morphological studies have shown that \(~75%\) of the vagal pulmonary afferents are C fibers (1). In the study of cultured neurons, the response was recorded from the neuronal soma, which precluded us from determining precisely the specific types of bronchopulmonary afferents of the individual neurons. However, one of the most prominent characteristics of pulmonary C-fiber afferents is their distinct sensitivity to capsaicin in vivo (13). Similarly, the percentage of isolated nodose and jugular neurons that respond to capsaicin is distinctly higher in cells of small diameter (<25 \(\mu\)M) than in large-diameter A neurons (2, 28). In this study, a majority (59.7\%) of the nicotine-sensitive pulmonary neurons was also activated by capsaicin (Fig. 6). In light of the recent finding that activation of NnAChRs by nicotine inhibits capsaicin-evoked inward current in isolated rat dorsal root ganglion neurons (7), the coexpression of sensitivity to nicotine and capsaicin in these vagal pulmonary sensory neurons suggests a possible interaction between these two chemical stimulants, and its potential...
implication of the role of nicotine in regulating the excitability of these sensory neurons is not clear.

Inhaled cigarette smoke evokes airway irritation accompanied by coughing and bronchospasm in both smokers and nonsmokers, resulting from an activation of sensory endings in the airways and lungs (18). Previous studies of the responses of all three major types of lung afferents to one or a few breaths of cigarette smoke in anesthetized rats (13) and dogs (16, 19) have shown that C fibers and RARs are most probable candidates involved in the airway irritation and cough reflex elicited by cigarette smoke. These previous studies also demonstrated that hexamethonium prevented the excitatory effect of cigarette smoke on these afferents but did not interfere with their normal responses to other chemical and mechanical stimuli (19), suggesting that the blocking effect is pharmacologically specific and nicotine is the causative agent. Results obtained from the in vivo experiments in the present study added further support to this hypothesis: right atrial injection of nicotine stimulated both pulmonary C fibers and RARs, but not SARs. Overall, a higher percentage of C-fiber afferents exhibited nicotine sensitivity than RARs in rat lungs, which is also consistent with our previous finding in the study of the stimulatory effect of cigarette smoke (13). In the present study of cultured pulmonary sensory neurons, pretreatment with hexamethonium abolished almost completely the Ca\(^{2+}\) transient evoked by nicotine (Fig. 4) but not their response to capsaicin or acid (data not shown). Furthermore, DMPP evoked a pattern of response similar to that of nicotine in the same neurons. Taken together, these results strongly suggest that the stimulatory effect of nicotine is mediated through an action on NnACHRs expressed on the membrane of these neurons. Study of the response of neuronal soma in culture also has its inherent limitations; for example, one key question is whether the same receptor proteins identified on the cell body, in this case the NnACHRs, are also expressed at the sensory terminals. However, this concern is lessened in the present study by the fact that similar stimulatory effects of nicotine and DMPP have also been demonstrated in the sensory endings in our in vivo experiments.

These results offer definitive evidence demonstrating that nicotine alone can activate the sensory endings in the airways and lungs, which is consistent with previous reports that nicotine administered by intravenous injection or aerosol inhalation can cause airway irritation and coughing in human subjects (12, 18). It has been extensively documented that various proinflammatory neuropeptides (substance P, calcitonin gene-related peptide, neurokinin A, etc.) are localized in the bronchopulmonary C-fiber afferent nerves in several species, including humans (15, 23). Stimulation of these sensory nerves by inhaled irritants, such as nicotine, not only elicits pulmonary reflexes and bronchocostriction via the brain stem reflex pathways (3, 21) but also triggers the release of these neuropeptides from the afferent endings in the airways, which are known to cause protein extravasation, bronchocostriction, mucus hypersecretion, and immunomodulatory effects (15, 23). Thus intense and prolonged stimulation of these sensory nerves can lead to the development of neurogenic inflammatory reactions in the lungs, particularly in rodents (15, 23). Whether the repetitive stimulation of these sensory endings by nicotine plays a part in the development of chronic airway diseases in smokers requires further investigation.

In conclusion, nicotine exerts a direct stimulatory effect on isolated rat vagal pulmonary sensory neurons, and the effect is probably mediated through an activation of the NnACHRs expressed on the membrane of these neurons. The specific subtypes of the NnACHRs mediating this response and their subunit compositions remain to be determined in these sensory neurons. Results obtained in this study further support our hypothesis that nicotine is primarily responsible for triggering cough and airway irritation evoked by inhaled cigarette smoke. Thus a possible involvement of the irritant effect of nicotine in the development of chronic airway diseases associated with smoking should not be overlooked.

GRANTS

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