Bimodal Concentration-Response of Nicotine Involves the Nicotinic Acetylcholine Receptor, Transient Receptor Potential Vanilloid Type 1, and Transient Receptor Potential Ankyrin 1 Channels in Mouse Trachea and Sensory Neurons

Tatjana I. Kichko, Jochen Lennerz, Mirjam Eberhardt, Ramona M. Babes, Winfried Neuhuber, Gerd Kobal, and Peter W. Reeh

Institute of Physiology and Pathophysiology (T.I.K., J.L., M.E., R.M.B., P.W.R.) and Institute of Anatomy I (W.N.), Friedrich-Alexander-University, Erlangen, Germany; Institute of Pathology, University of Ulm, Ulm, Germany (J.L.); Department of Biophysics, Carol Davila University of Medicine and Pharmacy, Bucharest, Romania (R.M.B.); Department of Anesthesiology and Intensive Care, Hannover Medical School, Hannover, Germany (M.E.); and Altria Client Services, Inc., Richmond, Virginia (G.K.)

ABSTRACT

High concentrations of nicotine, as in the saliva of oral tobacco consumers or in smoking cessation aids, have been shown to sensitize/activate recombinant transient receptor potential vanilloid type 1 (rTRPV1) and mouse TRPA1 (mTRPA1) channels. By measuring stimulated calcitonin gene-related peptide (CGRP) release from the isolated mouse trachea, we established a bimodal concentration-response relationship with a threshold below 10 μM (–)-nicotine, a maximum at 100 μM, an apparent nadir between 0.5 and 10 mM, and a renewed increase at 20 mM. The first peak was unchanged in TRPV1/A1 double-null mutants as compared with wild-types and was abolished by specific nicotinic acetylcholine receptor (nAChR) inhibitors and by camphor, discovered to act as nicotinic antagonist. The nicotine response at 20 mM was strongly pH-dependent, – five times greater at pH 9.0 than 7.4, indicating that intracellular permeation of the (uncharged) alkaloid was required to reach the TRPV1/A1 binding sites. The response was strongly reduced in both null mutants, and more so in double-null mutants. Upon measuring calcium transients in nodose/jugular and dorsal root ganglion neurons in response to 100 μM nicotine, 48% of the vagal (but only 14% of the somatic) sensory neurons were activated, the latter very weakly. However, nicotine 20 mM at pH 9.0 repeatedly activated almost every single cultured neuron, partly by releasing intracellular calcium and independent of TRPV1/A1 and nAChRs. In conclusion, in mouse tracheal sensory nerves nAChRs are 200-fold more sensitive to nicotine than TRPV1/A1; they are widely coexpressed with the capsaicin receptor among vagal sensory neurons and twice as abundant as TRPA1. Nicotine is the major stimulant in tobacco, and its sensory impact through nAChRs should not be disregarded.

Introduction

For smokers, nicotine not only exerts pharmacologic effects but is an essential part of the smoking experience, providing the desirable somatosensory impact and a distinct smell (Hummel et al., 1992). In smoking cessation aids, oral tobacco products, and in the “electric cigarette,” nicotine is a main sensory stimulant that can cause pleasantly mild burning but eventually unpleasant, pungent sensations (Einaron and Einaron, 1997; Jimenez Ruiz et al., 2000; Bolliger et al., 2007; Talavera et al., 2009). Such excitatory effects of nicotine derive from ubiquitous primary polymodal nerve endings, the subpopulation of which is equipped with nicotinic acetylcholine receptors (nAChR) (Steen and Reeh, 1993; Bernardini et al., 2001; Rau et al., 2005). The physiologic role of these nAChRs is not yet clear, although it has been speculated they may subserve pain induction when acetylcholine is set free from damaged epithelial cells, such as in the cornea (Tanelian 1991). Many epithelia contain acetylcholine (ACh) and the machinery to produce, store, and release it (Zia et al., 2000; Wessler and Kirpatrick, 2008). For example, in the mouse trachea, epithelium-derived bronchoconstriction is mediated by ACh, and skin keratinocytes release graded amounts of ACh upon stimulation by noxious heat or resiniferatoxin, an activator of the capsaicin receptor transient receptor potential vanilloid type 1 (TRPV1) (Moffatt et al., 2004; Nagy et al., 2006). Nicotinic ACh receptors have not remained the only known targets of nicotine on sensory nerves: TRPV1 was shown to be

ABBREVIATIONS: ACh, acetylcholine; AITC, allyl isothiocyanate; ANOVA, analysis of variance; BCTC, N-(4-t-butylphenyl)-4-(3-chloropyridin-2-yl) tetrahydroprazopyrazine-1(2H)-carboxamide; CGRP, calcitonin gene-related peptide; CHO, Chinese hamster ovary; DRG, dorsal root ganglion; HC030031, 2-(1,3-dimethyl-2,6-dioxo-1,2,3,6-tetrahydro-7H-purin-7-yl)-N-(4-isopropylphenyl) acetamide; LSD, least significant difference; MO, mustard oil; nAChR, nicotinic acetylcholine receptor; NJG, nodose/jugular ganglion complex; SIF, synthetic interstitial fluid; TRPA1, transient receptor potential ankyrin 1; TRPV1, transient receptor potential vanilloid type 1.
directly sensitized and the almost universal chemoreceptor-channel transient receptor potential ankyrin 1 (TRPA1) to be fully activated by nicotine (Liu et al., 2004; Talavera et al., 2009). Surrogate studies on cellular models even suggest an equal potency of nicotine to activate nAChR and TRPA1 (Rau et al., 2005; Talavera et al., 2009). However, a complete concentration-response relationship for nicotine on native sensory neurons, which may coexpress TRPV1, TRPA1, and nAChR, has not yet been established nor have different activating components been dissected. Up to high millimolar nicotine concentrations are contained, for example, in nasal sprays and dissolve in human saliva when oral tobacco products are used (Benowitz et al., 2009).

Nicotine is an alkaloid (pKa 8.02 at 37°C), and as such it is largely charged at pH 7.4, only fractions being available to permeate plasma membranes. This should not matter in the case of nAChR because its ligand-binding pockets are extracellular (Arias, 1997). However, TRPV1 and TRPA1 have intracellular binding sites for most of their ligands and thus should be more sensitive to nicotine in alkaline solution (Jung et al., 1999; Talavera et al., 2009).

Other means of discriminating the nicotine-activated receptor-channels are selective agonists and antagonists. However, the long-approved nicotinic antagonist mecamylamine has recently been shown to block TRPA1, although in hundreds of times higher concentrations than required for nAChR (Talavera et al., 2009). This side effect was not unexpected, as mecamylamine is a largely charged at pH 7.4, only fractions being available to permeate plasma membranes. This should not matter in the case of nAChR because its ligand-binding pockets are extracellular (Arias, 1997). However, TRPV1 and TRPA1 have intracellular binding sites for most of their ligands and thus should be more sensitive to nicotine in alkaline solution (Jung et al., 1999; Talavera et al., 2009).

The TRPA1 agonist acrolein has not yet been found to act through other TRP receptors; it is an endogenous product of lipid peroxidation, associated with neurodegenerative diseases, reperfusion injury, and chronic inflammation (Tsirulnikov et al., 2012). In addition, acrolein is a major constituent of smoke, in particular of the gas phase of cigarette smoke, and there it accompanies a variety of other volatile TRPA1 agonists, such as formaldehyde, acetaldehyde, and crotonaldehyde (Roemer et al., 2012).

A final aspect by which nicotine effects could be dissected is selectivity of action on particular subsets of sensory neurons. Here, we compare mouse dorsal root and nodose/jugular ganglion neurons in culture, the latter representing the cell bodies of vagal sensory nerve fibers. These neurons also provide the vast majority of sensory nerve endings in the trachea, the isolated tissue preparation used here to measure stimulated release of calcitonin gene-related peptide (CGRP) as an index of sensory activation and neurosecretion (Kichko and Reeh, 2009). However, CGRP release is also a (patho-)physiologic parameter of interest in its own right because it goes along with substance P release, and both neuropeptides together are a hallmark of neurogenic inflammation in the trachea and elsewhere (Brain, 1997; Myers et al., 2002). In this respect, our data show a wide segregation of specific nicotinic effects at micromolar concentrations from TRPV1/A1-dominated actions at higher millimolar levels which, in addition, become prevalent only at alkaline pH. Further, we show that vagal sensory neurons are much more sensitive to nicotine than somatic ones and that camphor is more sensitive to nicotine than TRPA1/V1 double knockout mice were used. Breeding pairs of TRPV1 and TRPA1 knockout mice were obtained from Dr. John Davis (Davis et al., 2000) and Dr. David Corey (Kwan et al., 2006) and were continuously backcrossed to C57BL/6. Double knockout animals were generated in our animal facility by cross-mating knockouts of both strains. The mice were housed in group cages in a temperature-controlled environment on a 12-hour light/dark cycle and were supplied with water and food ad libitum. Mice of either sex (body weight 15–25 g) were killed by exposure to pure CO2 atmosphere (approved by the Animal Protection Authority, District Government Mittelfranken, Ansbach, Germany).

Trachea Preparation. The trachea was excised together with the two main bronchi, and hemisectioned along the sagittal midline. One half of the trachea preparation was used as the control and the other half for chemical treatments, taking advantage from the minor individual variability of CGRP release. The preparations were exposed for 30 minutes at 37°C in carbogen-gassed (95% O2, 5% CO2, obtaining pH 7.4) synthetic interstitial fluid (SIF) containing (in mM) 107.8 NaCl, 3.5 KCl, 1.53 CaCl₂, 0.69 MgSO₄, 26.2 NaHCO₃, 1.67 NaH₂PO₄, and 9.64 sodium gluconate. After the initial rest period, the isolated trachea was consecutively incubated for 5 minutes in each of four tubes containing 125 µl SIF or stimulated chemicals, and was mounted in a shaking bath at 37°C. The first two incubations were performed to determine the basal CGRP release and variations at 37°C. The third tube contained the stimulating chemicals diluted in SIF (nicotine, epibatidine, acrolein, and mustard oil); when indicated, the antagonistic compounds were added. The final tube was used for washout and to check for reversal.

CGRP Enzyme Immunoassay. The CGRP content of the incubation fluid was measured using commercial enzyme immunoassay kits with a detection threshold of 2 pg/ml (Bertin Pharma, Montigny-le-Bretonneux, France). For this purpose, 100 µl of the sample fluid was stored on ice, and immediately after the trachea incubation period, it was mixed with 25 µl of 5-fold concentrated commercial CGRP enzyme immunoassay buffer that contained a cocktail of peptidase inhibitors. The CGRP contents were determined after the end of the experiment; the antibody reactions took place overnight. Enzyme immunoassay plates were determined photometrically using a microplate reader (Dynatech, Channel Islands, UK).

All results are presented as measured by the enzyme immunoassay in picogram of CGRP per milliliter of SIF. Reducing the interindividual variability and day-to-day baseline variability, the data were normalized to the second individual baseline value (before stimulation). This value was subtracted from all four data points of a typical experiment so that only the absolute change in CGRP release (A picogram per milliliter) is displayed in the figures.

Cell Culture. Nodose/jugular ganglia of both sides and dorsal root ganglia of lumbar and the first two thoracic segments of the spinal column of mice were excised and transferred into Dulbecco’s modified Eagle’s medium (DMEM) solution containing 50 µg/ml gentamicin (Sigma, Taufkirchen, Germany). The ganglia were treated with 1 mg/ml collagenase and 0.1 mg/ml protease (both from Sigma) for 30 minutes and subsequently were dissociated using a fire-polished silicone-coated Pasteur pipette. The cells were plated on poly-D-lysine-coated (200 µg/ml; Sigma) coverslips and cultured in TNB100 cell culture medium supplemented with TNB100 lipid-protein complex, 100 µg/ml streptomycin, penicillin (all from Biochrom, Berlin, Germany), and mouse NGF (100 ng/ml; Almone Laboratories, Tel Aviv, Israel) at 37°C and 5% CO2. Calcium imaging experiments were performed within 20 to 30 hours of dissociation.

Ratiometric [Ca²⁺], Measurements. Neurons of nodose/jugular or dorsal root ganglia of C57BL/6 and TRPV1/1–/– mice were stained by 3 µM Fura-2 acetoxyethyl ester (Fura-2 AM) and 0.02% pluronic (both from Invitrogen, Darmstadt, Germany) dissolved in TNB100 medium for about 30 minutes. After a 30-minute washout period to allow Fura-2-AM deesterification, the coverslips were mounted on an Olympus IX71 inverse microscope with a 10× objective.
The ganglion cells were constantly superfused with extracellular fluid (in mM: 145 NaCl, 5 KCl, 1.25 CaCl\(_2\), 1 MgCl\(_2\), 10 glucose, 10 HEPES) using a software controlled 7-channel gravity-driven common-outlet superfusion system. Fura-2 was excited at 340 and 380 nm with a Polychrome V monochromator (Till Photonics, Graefelfing, Germany). The images were exposed for 200 \(\mu s\) and were acquired at a rate of 1 Hz with a 12-bit CCD camera (Imageo Sensicam QE, Till Photonics). Data were recorded and further analyzed using TILLvisION 4.0.1.3 software (Till Photonics). The background was subtracted before the calculation of ratios.

All experimental protocols were preprogrammed. During experiments, the cells on the coverslip were exposed to different stimulations for 20 seconds each (capsaicin for 10 seconds). Between stimuli, the cells were washed with fresh extracellular buffer for 6 minutes to allow the cells to recover before the next stimulus. Capsaicin and mustard oil were used for cell categorization. At the end of all protocols, a 30-second stimulus of 60 mM KCl was applied to depolarize the cells as a control and as the normalization reference for comparison between the different stimulations. Ratio increases reflecting calcium concentration were calculated (Poenie and Tsien, 1986). An increase of the intracellular calcium concentration of at least 50 nM during the application period was considered as activation.

Stimulation responses were quantified as the area under the curve of the fluorescence ratio during the application period and S.E.M.; 10 seconds before application was used as the reference period.

**Chemicals.** Chemicals used in this study are as follows. From Sigma, we obtained (\(\geq\))camphor, capsaicin, mecamylamine hydrochloride, methyllycaconitine citrate hydrate, epibatidine, mustard oil (A1Tc, allyl isothiocyanate), acrolein, and the TRPA1 blocker HC030031, 2-(1,3-dimethyl-2,6-dioxo-1,2,3,6-tetrahydro-7H-purin-7-yl)-N-(4-isopropylphenyl) acetamide. From Acros (Geel, Belgium), we obtained (\(\geq\))-nicotine. From Biomol (Cologne, Germany), we obtained the TRPV1 inhibitor BCTC, N-(4-4-butyphyl)-4-(3-chloropyridin-2-yl) tetrahydroxyprazine-1(2H)-carboxamide.

Initial stock solutions were made in ultrapure H\(_2\)O except for camphor, capsaicin, BCTC, and mustard oil (made in 100% ethanol) and were stored at \(-24^\circ\)C. The final solutions ready to use were freshly diluted in SIF or external solution before each experiment. The camphor 2 mM solution contained 0.2% ethanol, and the other final solutions (BCTC, capsaicin, MO) contained 0.1% ethanol or less. A concentration of 20 mM nicotine overloaded the buffer capacity of the physiologic solution (generating pH \(-8.0\)) and required HCl titration to achieve pH 7.4 and NaOH for pH 9.0.

**Statistical Analysis.** Statistical comparisons were performed using Statistica 7 software (Statsoft, Tulsa, OK). All time series of experimental values were first analyzed for the effect of stimulation (by nicotine, epibatidine, acrolein, MO) as compared with baseline using the nonparametric Wilcoxon matched pairs test. The baseline-normalized (i.e., \(\Delta\)) CGRP values were entered into a one-way analysis of variance (ANOVA) followed by Fisher’s least significant difference (LSD) test, focusing on the peak values of stimulated CGRP release. \(P < 0.05\) was considered statistically significant (and is labeled with an asterisk throughout). Data points represent the mean ± SEM of the given number (n) of experiments.

**Results**

\(-\)nicotine-induced tracheal CGRP release shows a bimodal concentration response. The tracheal nicotine concentration-response in wild-type (C57Bl/6) mice showed a statistically significant increase over baseline CGRP secretion at 10 \(\mu M\) (\(-\))nicotine, a maximum at 100 \(\mu M\) (pH 7.4), an apparent nadir between 0.5 and 10 mM, and a second increase with 20 mM (Fig. 1A). A log-linear rising flank up to 100 \(\mu M\) provided a half-maximal effective concentration (EC\(_{50}\)) of about 31 \(\mu M\); significantly less stimulated CGRP release was achieved at supramaximal concentrations of 500 \(\mu M\) and 10 mM in comparison with 100 \(\mu M\). This inversely U-shaped relationship suggested an inhibitory action of nicotine concentrations \(>100 \mu M\) and \(<20 \mu M\).

This hypothesis was tested using the ultrapotent and specific nicotinic agonist epibatidine (0.1 \(\mu M\)). Its effect was neither enhanced nor reduced by coapplication of 1 mM nicotine (n = 8, data not shown), making improbable a block of the ion channel as well as a heterologous desensitization of the nAChR. However, a homologous desensitization, typical for nAChRs, became evident when the nicotine (100 \(\mu M\)) exposure was extended from 5 to 10 minutes, and the CGRP release induced during the first 5 minutes returned to baseline during the second 5 minutes (n = 4, data not shown).

A further typical property of nAChRs is their absolute enantiomer-selective preference for \((\pm)\) versus \((\pm)\)-nicotine, the latter of which (100 \(\mu M\)) did not evoke any tracheal CGRP release (n = 4, data not shown). Release of CGRP by vesicular exocytosis requires calcium entry into the sensory nerve fibers. Accordingly, in the presence of EGTA (10 mM) and absence of extracellular calcium the basal (constitutive) CGRP release from the trachea was significantly reduced (not shown), and no nicotine response at maximal effective concentration of 100 \(\mu M\) could be observed (Fig. 1A), which indicates a physiologic mechanism of neurosecretion.

Double-knockout TRPA1/TRPV1\(^{\mathrm{−/−}}\) mice, congenic with C57Bl/6, were used to exclude the participation of these TRP channels in the assumed specific nicotinic response of the

---

**Fig. 1.** (A) Concentration-dependent nicotine-induced CGRP release from isolated trachea of wild-type mice. The threshold concentration is 10 \(\mu M\) (statistically significant over baseline, Wilcoxon test), EC\(_{50}\) \(\sim 31 \mu M, P < 0.01\) and smaller applies to one-way ANOVA followed by LSD Fisher test. In the presence of EGTA (10 mM) and absence of extracellular calcium ions, no response to maximal effective nicotine concentration (100 \(\mu M\)) was observed. (B) Specific nicotinic responses in congenic wild-type (C57Bl6) mice and TRPA1/V1 double-null mutants. (Data are normalized to baseline, i.e., the second incubation/sampling period.)
trachea. The stimulated CGRP release at 31 and 100 μM nicotine concentration was the same in knockout as in wild-type mice (Fig. 1B), suggesting that the lower-concentration mode of the nicotine actions is mediated by nACHRs, which will be demonstrated later.

**Specific Micromolar Nicotinic Actions.** The pharmacology of lower-concentration nicotine-induced tracheal CGRP release was in accord with the knockout findings. The classic unselective nACHR antagonist hexamethonium abolished the nicotine response (Fig. 2A). Mecamylamine, the more selective antagonist of the ganglionic, heteromeric nACHRs, showed concentration-dependent efficacy, while methyllycaconitine, preferring the CNS type of nACHR, was ineffective. This suggests an α-bungarotoxin-insensitive subunit composition of the heteropentameric nACHR subtype, most likely αβγδ, in agreement with immunocytochemical findings on tracheal sensory nerves (Haberberger et al., 2004).

In contrast with the nicotinic blockers, the specific TRPV1 antagonist BCTC (Valenzano et al., 2003) and the TRPA1 antagonist HC030031 (Eid et al., 2008) did not diminish the tracheal nicotinic response (Fig. 2B), while the latter was effective against the selective TRPA1 agonist acrolein, another essential constituent of tobacco smoke (Fig. 2C). Mecamylamine was also tested against acrolein because it had recently been shown to inhibit TRPA1 in a much higher concentration (Talavera et al., 2009); this, however, was not the case at 100 μM equimolar to nicotine (Fig. 2C). To exclude any sensitizing effect of lower nicotine concentration on TRPA1 (Talavera et al., 2009), nicotine was coapplied with the TRPA1 agonists acrolein and MO (AICt) at low, TRPA1-selective concentrations. The CGRP-releasing effects were solely additive (Fig. 2D), suggesting independence of each other of nicotinic and TRPA1-mediated actions.

**Antinicotinic Effects of Camphor.** An interesting overlap of the antagonist profiles of TRPA1 and nACHR was discovered using camphor. This monoterpenoid, structurally similar to mecamylamine, is an established TRPA1 antagonist (Xu et al., 2005) and was not previously known to block nicotine effects on nACHR (Park et al., 2001). Although camphor is a chiral substance, both enantiomers (+ and −) at 2 mM were equally effective (data not shown) abolishing the specific nicotine responses in the trachea (Fig. 2B) as well as the acrolein effect (Fig. 2C). This antinicotinic camphor effect was concentration-dependent in a narrow range between 0.2 and 1 mM upon coapplication with nicotine at half-maximal effective concentration (Fig. 3A).

The novel action of camphor appeared worthy to be reproduced in an independent model using cultured sensory neurons from the nodose/jugular ganglion (NJG) complex, part of which innervate the trachea and respond with calcium influx to sensory irritants such as MO and capsaicin as well as to nicotine (further details see below). In this cellular model camphor also abolished the specific nicotinic response in a reversible manner and without inducing calcium influx by itself (Fig. 3B). Further support for a specific antinicotinic action of camphor came again from the trachea, where it inhibited the CGRP release evoked by epibatidine, although not as effectively and by far not as potently as mecamylamine (Fig. 3C).

These data also confirmed the high selectivity of epibatidine for nACHR, because both TRPV1 and TRPA1 null mutants showed about the same nicotinic responsiveness as the congenic wild types (Fig. 3, C and D). A saturating concentration of epibatidine (0.1 μM) evoked the same amount of tracheal CGRP release as the maximal effective nicotine concentration (100 μM, Fig. 1A), confirming the 1000-fold greater potency of epibatidine over nicotine (Bradley, 1993) and indicating that all accessible neuronal AChRs had been activated by either agonist. Camphor showed its full inhibitory effect on epibatidine also in TRPA1 knockouts (Fig. 3D), finally confirming that its antinicotinic action was not due to a TRPA1 block.

**Multiple Actions of Millimolar Nicotine.** The high concentrations (in millimolars) of nicotine that occur in the saliva of consumers of oral tobacco products and nicotine-containing chewing gums may contribute to the harshness of strong cigarettes as perceived on the laryngeal level (Lee et al., 1987; Talavera et al., 2009; Stanfill et al., 2011). TRPV1 and TRPA1 had previously been identified as mediators of high-concentration neuronal nicotine effects (Liu et al., 2004; Talavera et al., 2009). Nicotine-induced tracheal CGRP release confirmed this involvement of TRP channels by showing a deficit in nicotine (20 mM) responsiveness in TRPA1 and a complete loss in double-mutants (Fig. 4A). Although these deficits were evident at pH 7.4 of the extracellular nicotine solution, they were much more prominent at pH 9.0 when the alkaloid is largely unchanged (pK₈, 8.02; Lu, Chen, Zhan, 2007) and, thus, well membrane-permeable. This is most likely due to the fact that both TRP receptor-channels have their binding sites for most agonists at the intracellular site of the transmembrane proteins (Vriens et al., 2009). Thus, the much greater efficacy of nicotine in alkaline than neutral solution (in C57BL/6) is most likely the result of a higher intracellular concentration.

SIF at pH 9.0 (titrated by NaOH) did not stimulate any CGRP release (n = 7, data not shown). The double-knockout mice for both TRPA1 and TRPV1 showed further reduced nicotine responsiveness, and hexamethonium caused an additional reduction in these animals (Fig. 4A), which is suggestive for a small but specific nicotinic component that was masked in wild types (in which 3 mM hexamethonium was ineffective against the high nicotine concentration; n = 6, data not shown). Enantiomer-selectivity of the high-concentration (20 mM) alkaline nicotine effects on TRP channels was not expected but, to our surprise, was confirmed as only 25% of the control CGRP release was evoked using (+)-nicotine (n = 4, data not shown). Hence, both TRP channels show a clear preference for the naturally occurring (−)-nicotine but not as exclusive as the nACHRs do (see above).

Even in double-knockouts and in the presence of hexamethonium, nicotine 20 mM at pH 9.0 caused a small but significant increase of tracheal CGRP release over baseline; this effect appeared worth scrutinizing in the cellular model of cultured sensory neurons (Fig. 4, B–D). NJG and DRG neurons of the TRPA1/V1 double-knockout mice were used for calcium imaging experiments. As expected, both neuron types did not respond to MO at a concentration (1 mM) that would activate TRPA1 as well as TRPV1 in wild types (Everaerts et al., 2011). However, nicotine 20 mM at pH 9.0 activated every single one of the tested double-knockout neurons to an extent that was not significantly different from wild-type neurons (Figs. 6 and 7); extracellular solution at pH 9.0 did not stimulate any calcium influx by itself. Hexamethonium did not reduce this...
cellular nicotine response (Fig. 4D) in contrast to the residual tracheal response (Fig. 4A).

However, in nominally calcium-free (+EGTA) extracellular solution, the nicotine response was reversibly diminished by 75% though not abolished (Fig. 4D). Thus, uncharged membrane-permeable nicotine in high concentration appears to activate another calcium entry mechanism, perhaps another TRP channel besides nAChR, TRPV1, and TRPA1; in addition, it is able to release calcium from intracellular stores. This intracellular calcium increase is obviously reversible, repeatable, pH-dependent, and thus not likely of cytotoxic nature. The unknown calcium influx mechanism may account for the (however small) residual nicotine-induced tracheal CGRP release in the double-knockouts and the presence of hexamethonium (see above), because unmyelinated nerve fibers and endings require calcium influx for vesicular exocytosis in any case as they do not possess any appreciable calcium stores.

The difference between nerve terminals bare of endoplasmic reticulum and sensory nerve cell bodies with abundant endoplasmic reticulum may account, in part at least, for the discrepancy between the small residual CGRP release from the former and the full-blown calcium transient in the latter. Accordingly, this discrepancy did not occur at pH 7.4 when nicotine (20 mM) is largely membrane impermeable (see below Figs. 5A and 6C).

**pH Dependence of the Cellular Nicotine Response.** The strong pH dependence seen with nicotine (20 mM)-induced tracheal CGRP release was scrutinized using NJG neurons of wild-type mice and calcium imaging. Alkaline pH more than doubled the prevalence of nicotine responsive cells and the magnitude of the calcium transients as compared with 20 mM nicotine at pH 7.4 (Fig. 5A). MO responses at TRPA1-specific concentration (100 μM) were clearly depressed by a preceding strong nicotine stimulus, most likely due to cross-desensitization of TRPA1 (Ruparel et al., 2008; Talavera et al., 2009). If the order of stimuli was reversed, the MO response was obviously greater and exceeded the nicotine response (Fig. 5B). Subsequent capsaicin responses did not appear to be much affected by the preceding stimuli. The pH, whether neutral or alkaline, did not matter if the nicotine concentration was only 1 mM (Fig. 5C), suggesting that even this relatively high concentration was still predominantly acting through nAChRs, which bind nicotine at the extracellular site of the receptor channel.

**Vagal NJG versus Spinal DRG Neurons.** It was the specific nicotinic (100 μM) responsiveness that distinguished DRG from NJG neurons, which innervate almost only internal organs including larynx and trachea. Prevalence and magnitude of the stimulated calcium transients were significantly greater among the vagal versus spinal sensory neurons (Table 1), and the pH of the nicotine solution did not matter at a 100 μM concentration (Fig. 6, A and B). Nicotine 100 μM also did not exert appreciable heterologous desensitizing were ineffective. (C) The selective TRPA1 agonist acrolein caused concentration-dependent tracheal CGRP release with a minimal effective concentration of 10 μM. The TRPA1 blockers HC030031 and camphor reduced the acrolein responses, whereas mecamylamine was ineffective. (D) The half-maximal effective nicotine concentration coapplied with low TRPA1-selective concentrations of mustard oil (AITC) and acrolein showed only additive effects.
Fig. 3. Specific antinicotinic camphor effects. (A) Camphor concentration-dependently decreased the half-maximal tracheal nicotinic response normalized to baseline. The insert shows the percentage reduction of CGRP release upon coapplication of nicotine and camphor. (B) Camphor reversibly abolished nicotinic-induced calcium influx in cultured sensory neurons from the mouse nodose/jugular ganglion complex and did not show stimulatory effect by itself. Mustard oil (MO = AITC), capsaicin, and KCl were positive controls for TRPA1, TRPV1 activation, and unspecific depolarization, respectively. (C) Camphor and mecamylamine reduced the tracheal response to the ultrapotent nicotinic agonist epibatidine coapplied at saturating concentrations and equally effective in wild-type and TRPV1<sup>−/−</sup>. (D) Inhibitory camphor effect in TRPA1<sup>−/−</sup> on the tracheal epibatidine response. (P values are one-way ANOVA followed by Fisher’s LSD test.)

Fig. 4. Supramaximal (20 mM) nicotine effects depend on genotype and pH. (A) Tracheal CGRP release at neutral and alkaline pH involving TRPA1, TRPV1, nAChR channels. The small but significant responses retained in double-knockouts (at pH 9.0) are reduced by hexamethonium. (B–D) Averaged calcium transients (± S.E.M. in sensory neurons of TRPV1/1A1 double-null mutants. (B) Vagal NJG neurons respond to nicotine but not MO (AITC) and pH 9.0 (left panel). Areas under the curve (AUC) show the response magnitudes and percentages of neurons responding (right panel). (C) Somatic DRG neurons. (D) DRG neurons without extracellular calcium showed reduced percentage and AUC upon 20 mM nicotine at pH 9.0, partial recovery upon calcium return, and no effect with hexamethonium (10 mM). The columns (right panel) are ordered according to the sequence of stimuli. (P values one-way ANOVA followed by Fisher’s LSD test.)
effects on the subsequent MO responses. In contrast, such as desensitization was obvious after 20 mM nicotine at pH 9.0 (Fig. 6C). The high prevalence (up to 99%) and the large magnitude of these supramaximal nicotine responses in wild-type DRG neurons were neither different from NJG cells nor from DRGs of the TRPV1/A1 double-knockouts, which again emphasizes the unspecificity of the high nicotine concentration (Table 1). Interestingly, the accentuated concentration-response relationship seen with nicotine-induced tracheal CGRP release (Fig. 1A) did not show up in NJG neurons. Nicotine (100 μM, 1 mM, and 20 mM, pH 9.0) evoked about the same submaximal increases of intracellular calcium (compare Figs. 5, A and D, and 6A).

**Distribution of Chemosensitivities among Sensory Neurons.** Chemosensory-induced calcium transients were used to compute Venn diagrams that illustrate the prevalences and their overlap among cultured NJG and DRG neurons at different nicotine and associated pH levels (Fig. 7). Capsaicin (0.3 μM) and MO (100 μM) sensitivities were about evenly distributed over four different random samples of neurons from both types of ganglia (n = 442). Capsaicin activated 48% and MO 25%, on average. Among NJG neurons, the specific nicotine sensitivity ranged with the high prevalence of capsaicin responsiveness, although in general the overlap between the chemosensory subpopulations was great but not complete; each chemical activated a number of neurons that were not sensitive to either of the other agents. Among DRG neurons, the specific nicotinic responses were rare (and small), but 20 mM nicotine (at pH 7.4) activated as many cells as capsaicin and MO together, and finally all neurons if the nicotine solution was alkalinized (pH 9.0). The maximized prevalence of nicotine (20 mM) responsiveness at pH 9.0 in DRG as well as in NJG neurons comprised most of the previously chemosensitive cells, but even more those units that had before appeared insensitive to all three chemicals.

This finally supports the conclusion (from Fig. 4D) that intracellular calcium release and some unknown calcium entry mechanism are activated by 20 mM nicotine at pH 9.0 in addition to nAChR, TRPV1, and TRPA1. The contributions of these identified receptor-channels were completely masked in the somata of sensory neurons by those unknown mechanisms of nicotine-induced intracellular calcium increase that were predominant in the cellular model. In the trachea, however, the contributions of the two TRP channels were evident, those of the nAChRs were small but discernible, and in the absence of the TRPs the CGRP release in response to 20 mM (pH 7.4) nicotine was abolished. Thus, if such a high concentration of nicotine ever reached the intraepithelial nerve endings in the trachea, neurogenic inflammation would mainly be mediated through TRPV1 and TRPA1. However, a 200 times lower nicotine concentration could still exert pronounced irritancy in the airways through nAChRs.

**Discussion**

Measuring stimulated CGRP release from the isolated mouse trachea, a bimodal concentration-response relationship was established with a threshold below 10 μM, an EC50 of
31 μM, a maximum at 100 μM (−)-nicotine, an apparent nadir between 0.5 and 10 mM, and a renewed increase at 20 mM. Using TRPV1, TRPA1, and double-null mutants as well as pharmacological tools, we could attribute the micromolar activity range to nAChRs; the higher millimolar range was dominated by activation of TRPV1 and TRPA1 in particular at alkaline pH when nicotine is uncharged and membrane permeable. The (+)-nicotine enantiomer was ineffective at low and weakly effective at high concentrations. The TRPA1 blocker camphor turned out to also inhibit nAChR, specifically though not selectively. Calcium imaging on cultured sensory neurons confirmed most of the results—except for the apparent nadir of the concentration response—and revealed that the vagal NJG neurons exhibit much greater specific nicotinic sensitivity than the somatic (i.e., DRG) neurons. However, all neuronal cell bodies of both innervation territories showed the same large responses upon supramaximal 20 mM nicotine at unphysiologic pH 9.0 in double-knockout and wild-type mice, suggesting a predominant unspecific effect that was partly due to intracellular calcium release.

The nicotine EC_{50} in our trachea preparation was as low as 31 μM. From recombinant rat nAChR composed of α3β4 subunits, EC_{50} values between 31 and 106 μM nicotine have been reported, whereas murine TRPA1-overexpressing Chinese

### Table 1
Comparison of NJG versus DRG neurons of wild-type vs. TRPV1/A1−/− mice at specific and supramaximal nicotine concentrations

<table>
<thead>
<tr>
<th></th>
<th>Nicotine, 100 μM, pH 7.4</th>
<th>Nicotine, 20 mM, pH 9.0</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% Prevalence</td>
<td>AUC × 100</td>
<td>% Prevalence</td>
</tr>
<tr>
<td>NJG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>48</td>
<td>25 ± 4a</td>
<td>86</td>
</tr>
<tr>
<td>TRPV1/A1−/−</td>
<td>24</td>
<td>8.5 ± 2.8</td>
<td>100</td>
</tr>
<tr>
<td>DRG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>14</td>
<td>6 ± 1.6a</td>
<td>99</td>
</tr>
<tr>
<td>TRPV1/A1−/−</td>
<td>1</td>
<td>0.7 ± 0.15</td>
<td>95</td>
</tr>
</tbody>
</table>

*Statistically significant difference between labeled numbers (P < 0.01, one-way ANOVA followed by LSD test).

Fig. 6. Prevalence and magnitude of specific nicotinic responsiveness greater in NJG than DRG neurons. (A) NJG neurons, calcium transients in response to specific nicotinic concentration about equal at pH 7.4 and 9.0, high percentage of cells responding, no cross-desensitization of MO (AITC) responses. (B) DRG neurons, low percentage of cells responding with small calcium transients, significantly smaller than NJG neurons (in A), #P < 0.01, one-way ANOVA followed by LSD test. (C) Almost all DRG neurons responded with large calcium transients to supramaximal nicotine at pH 9.0, less so at pH 7.4, and showed pronounced cross-desensitization against MO (AITC).
in between DRG and NJG with respect to nicotinic sensitivity. (Fig. 4, B and C), suggesting that trigeminal neurons might range by 100 neurons from TRPV1/TRPA1 double-null mutants were activated nicotinic action in this micromolar range. In fact, 24% of our NJG application of a selective antagonist strongly indicate a specific concentration-dependent increase and decrease by thonium, the specific nAChR blocker (Talavera et al., 2009). This 1 mM nicotine; this percentage dropped to 1.6% under hexame-

However, in TRPA1 null mutants, 4% of cultured trigeminal expression systems (Rau et al., 2005; Talavera et al., 2009). The phenomenon has unanimously been attributed to relationships of nicotinic and TRPA1 receptors in heterologous systems (Fucile et al., 2005). The fractional calcium conductance of voltage-gated calcium channels coexpressed in sensory neurons — as opposed to homopentameric — is restricted, but if they depolarize the neuron by sodium influx, those calcium channels will be activated and will provide cytosolic calcium increase and eventually CGRP release (Stetzer et al., 1996; Letz et al., 1997; Fucile et al., 2005).

In contrast to the cellular model, the nicotine-induced tracheal CGRP release showed this accentuated apparent nadir around 1 mM in the concentration-response curve that clearly separated a specific nicotinic, micromolar mode of action from the TRPV1/TRPA1-dominated millimolar mode, shaping an inverted U in the lower micromolar range. Effects that decrease or revert into the contrary at supramaximal concentration or dosage are a classic hallmark of the nicotine concentration/dose-response relationship and have been demonstrated in many animal and human experimental models (Ashton et al., 1980; Nakamura et al., 1986; Clarke 1990; Perkins et al., 1994; Krebs et al., 1994; Rowell and Li, 1997; Picciotto 2003; Matta et al., 2007). The phenomenon has unanimously been attributed to the well-known fast and complete homologous desensitization/tachyphylaxis of the nAChR that is particularly evident at high agonist concentrations (Jinks and Carstens 1999; Liu and Simon 1996; Dessirier et al., 2000; Rau et al., 2005). Our results from the trachea confirmed the principle of nicotinic desensitization, but this did not seem to explain the significant loss of efficacy at supramaximal, 0.5 and 10 mM, nicotine concentration, because the (maximal) epibatidine response was not reduced by additional 1 mM nicotine. Perhaps the low concentration (0.1 μM) of this ultrapotent agonist was still too high, creating a complete and prolonged nACh receptor occupancy so that (the 1000-fold) lower affinity nicotine passed by ineffectively.

To activate recombinant TRPA1 (in CHO cells), the nonelectrophilic nicotine binds to an as-yet-unknown but definitely intracellular site of the ion channel (Talavera et al., 2009), which is consistent with the pronounced increase of nicotine effects at unphysiologic alkaline pH in both of our models because only deprotonated nicotine can freely pass plasma membranes. On the other hand, nicotine is also reported to block the open TRPA1 pore at higher concentrations with an IC50 of 2 mM (Talavera et al., 2009). This open channel block was discovered using neutral nicotine solutions, when most of the alkaloid molecules are positively charged and prone to attach to negative charges in the channel walls. Hence, the blocking side effect is most likely reduced with alkaline nicotine solution, and uncharged nicotine may even pass the TRPA1 (and TRPV1) channel that is known for its unusually wide pore, conducting even larger molecules when activated (Karashima et al., 2010). Intracellularly, nicotine would then become protonated and thus trapped. Thereby, 20 mM nicotine—as in our experiments—conveys a considerable buffer capacity that would alkalize the axoplasm, which is a further condition activating TRPA1 (Fujita et al., 2008). All these augmenting and interlaced mechanisms together may explain the pronounced pH dependency of millimolar nicotine actions and account for the drastic, though not simply cytotoxic, effects of alkaline nicotine. At physiologic pH, these TRPA1/TRPV1-mediated and partly unspecific nicotine effects were much smaller and actually were smaller than the specific, genuinely nicotinic micromolar effects in NJG neurons as well as in the trachea.
The TRPA1 antagonist camphor is contained in numerous consumer products such as teas, liqueurs, spice blends, sweets, incense, snuff, and herbal cigarettes together with or without tobacco. Its role is to scent or flavor, and its smell is eucalyptus-like though in higher concentration is like mothballs (“camphor ball”). In medicinal liniments and ointments, camphor is contained in high concentrations, which are restricted by the U.S. Food and Drug Administration to 11% (FDA, 1982). Thus, within certain dose limits camphor can be generally regarded as safe. Therefore, it could be added to oral nicotine-containing products such as smoking cessation aids when sensory irritation becomes a problem that reduces compliance. Camphor could reduce the pungency of these products, first by blocking TRPA1 and second by blocking nAChRs of oral nerve endings. The suitable range of camphor concentrations appears limited to below 6 mM, because at this and higher levels camphor evoked CGRP release by itself (from isolated mouse buccal mucosa) and augmented the nicotine (30 mM, pH 9.0) response, both effects mediated through TRPV1 in TRPA1 null-mutants (T. Kichko and P. Reeh, unpublished data) (Xu et al., 2005). Decreasing the saliva pH by acidic nicotine-based oral smoking cessation aids would be another way of preventing burning mouth sensations, according to our results. However, neutral or lower pH also reduces the nicotine absorption, probably the speed of absorption if not the amount. Thus, the peak plasma concentrations that are required to assuage the craving for a cigarette may not be reached (Tomar and Henningfield, 1997).

When TRPA1 activation by nicotine was uncovered, a study demonstrated the quasi-physiologic relevance with a whole-animal experiment: 60 mM nicotine solution applied by nasal instillation resulted in a mild (compared with methacholine) bronchoconstrictory response of wild-type but not TRPA1-deleted mice. They did not test TRPV1 knockouts, but recombinant human TRPV1 was reported to be blocked rather than activated by 1 mM nicotine (Talavera et al., 2009). However, before that study, direct sensitization, but not activation, of rat TRPV1 in CHO cells by 0.1 and 1 mM nicotine had been reported (Liu et al., 2004). Our results from the native mouse trachea are at variance with both reports: the robust effect of 0.1 mM nicotine involved neither TRPV1 nor TRPA1 but rather nAChR (Fig. 1B), and high millimolar nicotine engaged all three receptor channels (Fig. 4A). The discrepancies might relate to species differences (rat/mouse), but they point to the principal differences between heterologous overexpression systems, cell culture models, and native tissues with intact innervation. Neither the nicotine concentrations affecting the tracheal mucosa and reaching its intraepithelial free nerve endings (Hauser-Kronberger et al., 1997) nor the local pH resulting from cigarette smoke or nicotine aerosol (“electric cigarette”) inhalation are known. However, even if TRPV1/TRPA1-activating millimolar nicotine concentrations together with alkaline pH were not attained, the much more sensitive and effective nAChRs—mostly coexpressed with substance P/CGRP in sensory neurons (Rau et al., 2005)—could still mediate neurogenic inflammation in the trachea with plasma extravasation and vasodilatation, as previously demonstrated by antidromic stimulation of vagal sensory nerves (McDonald et al., 1988). In addition, nicotine largely contained in the particulate phase of cigarette smoke comes together with a multitude of volatile TRPA1 agonists in the gaseous phase such as acrolein, formaldehyde, acetaldehyde, and crotonaldehyde (Roemer et al., 2012). André et al. (2008) have shown that the entirety of the water-soluble cigarette smoke constituents, which include nicotine, can activate TRPA1 in the mouse trachea. As specific nicotinic and TRPA1-activating effects are additive (Fig. 2D), the significance of the more sensitive nAChRs for sensory irritation should not be disregarded. Moreover, it is largely the same sensory neurons (Fig. 7) that mediate specific nicotinic as well as TRPV1 and TRPA1 agonistic actions; it is likely they send signals of sensory impact to the brain that are differently perceived by smokers and nonsmokers.

Acknowledgments

The authors thank Susanne Haus-Oertelt, Iwona Izdryczky, and Annette Kuhn for excellent technical assistance; Jana Schramm for genotyping and breeding the mutant mice; and Andrea Link for quantitative RT-PCR analysis.

Authorship Contributions

Participated in research design: Kichko, Reeh, Kobal.
Conducted experiments: Kichko, Babes.
Contributed new reagents or analytic tools: Lennerz, Neuhuber, Reeh.
Performed data analysis: Kichko, Eberhardt.
Wrote or contributed to the writing of the manuscript: Kichko, Reeh, Kobal.

References

Nicotine Responses in Trachea and Sensory Neurons 539


Address correspondence to: Dr. Peter W. Reeh, Department of Physiology and Pathophysiology, Friedrich-Alexander-University Erlangen-Nuremberg, Universitaetsstrasse 17, D-91054 Erlangen, Germany. E-mail: reeh@physiologie1.uni-erlangen.de