Cellular Basis for the Olfactory Response to Nicotine

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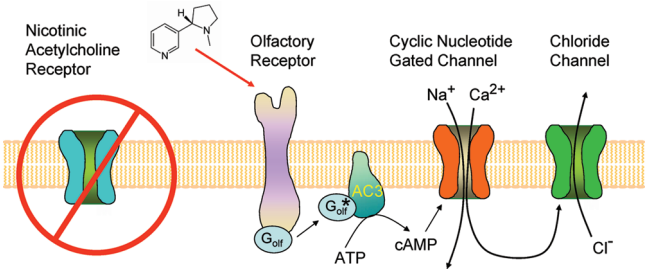
Abstract



Smokers regulate their smoking behavior on the basis of sensory stimuli independently of the pharmacological effects of nicotine (Rose, J. E., et al. (1993) *Pharmacol., Biochem. Behav. 44* (4), 891−900). A better understanding of sensory mechanisms underlying smoking behavior may help to develop more effective smoking alternatives. Olfactory stimulation by nicotine makes up a considerable part of the flavor of tobacco smoke, yet our understanding of the cellular mechanisms responsible for olfactory detection of nicotine remains incomplete. We used biophysical methods to characterize the nicotine sensitivity and response mechanisms of neurons from olfactory epithelium. In view of substantial differences in the olfactory receptor repertoire between rodent and human (Mombaerts, P. (1999) *Annu. Rev. Neurosci. 22*, 487−509), we studied biopsied human olfactory sensory neurons (OSNs), cultured human olfactory cells (Gomez, G., et al. (2000) *J. Neurosci. Res. 62* (5), 737−749), and rat olfactory neurons. Rat and human OSNs responded to S(−)-nicotine with a concentration dependent influx of calcium and activation of adenylate cyclase. Some rat OSNs displayed some stereoselectivity, with neurons responding to either enantiomer alone or to both. Freshly biopsied and primary cultured human olfactory neurons were less stereoselective. Nicotinic cholinergic antagonists had no effect on the responses of rat or human OSNs to nicotine. Patch clamp recording of rat OSNs revealed a nicotine-activated, calcium-sensitive nonspecific cation channel. These results indicate that nicotine activates a canonical olfactory receptor pathway rather than nicotinic cholinergic receptors on OSNs. Further, because the nicotine-sensitive mechanisms of rodents appear generally similar to those of humans, this animal model is an appropriate one for studies of nicotine sensation.

Keywords: Odor, tobacco, smoking, adenylate cyclase, cyclic nucleotide gated channel, transduction

While the pharmacological properties of nicotine have been studied extensively, less is known about the complex sensory qualities of nicotine as an odor. Sensory cues accompanying cigarette smoking contribute significantly to the smoker's satisfaction (*3*) and reinforcement of smoking, particularly among women (*4*). Concurrent airway sensory stimulation increases the effectiveness of smoking cessation products that deliver nicotine in other forms, for example, skin patch (*5*), and subjects appear to regulate their smoking behavior on the basis of sensory stimuli independently of the pharmacological effects of nicotine (*1*). Nicotine has a considerable influence on the aroma and flavor of tobacco smoke (*6*). Moreover the initial sensory impact and reinforcing value of denicotinized cigarettes is less than those of cigarettes containing nicotine (*7*). Since nicotine elicits odor sensations, olfactory stimuli may serve as part of the conditioned stimuli associated with the central nervous system pharmacological effects of nicotine. Thus, elucidation of the underlying sensory mechanisms may lead to more effective smoking alternatives.

Olfactory reception of nicotine is not well understood. Nicotine has been described as having a sweet, warm, or spicy odor at low to moderate concentrations (*8*). While the enantiomers of nicotine can be discriminated by humans (*8*, *9*), the receptor basis of this is not known. Human pharmacological studies suggest that the receptors are not nicotinic acetylcholine receptors because neither detection thresholds nor intensity ratings for the odor of nicotine were affected by the nicotinic acetylcholine receptor (AChR) noncompetitive antagonist mecamylamine (*10*). While these results suggest that nAChRs do not play a role in olfactory responses to nicotine in mammals, the study did not rule out the possibility of incomplete access of inhibitor to the olfactory sensory epithelium. Thus, the mechanisms

Received Date: December 22, 2009
Accepted Date: February 16, 2010
Published on Web Date: February 26, 2010

DOI: 10.1021/cn900042c | *ACS Chem. Neurosci.* (2010), *1*, 246–256

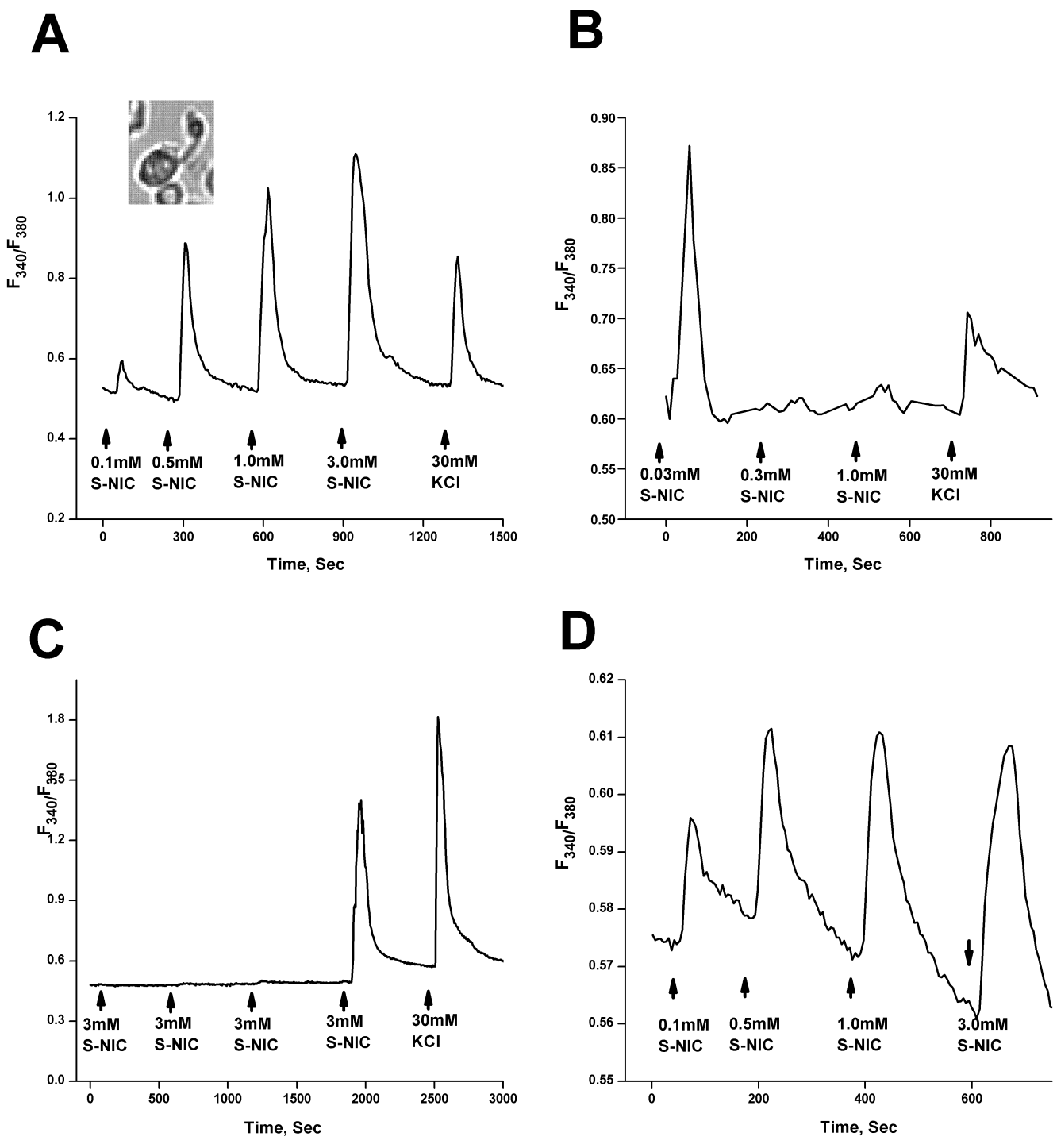


Figure 1. *S*(−)-Nicotine induces increases in intracellular calcium $[Ca^{2+}]_i$ in rat OSNs. Neurons showed a graded increase in response magnitude with increasing concentrations. (A; $n = 5$ of 6480 KCl-sensitive cells tested). The trace in panel a was obtained from the rat OSN shown in the inset. This morphology was typical of both rat and human olfactory neurons. In other rat OSNs, the response desensitized at low concentrations (B) or responded only after several single concentration stimulations (C). Cultured human olfactory cells responded similarly to *S*(−)-nicotine (D). Arrows indicate when the stimulus delivery valve was activated.

underlying the olfactory detection of nicotine remain unclear.

The present experiments examined the cellular basis of olfactory responses to nicotine using *in vitro* studies of human olfactory neurons, as well as neurons from an animal model, the rat olfactory system. This comparative approach sought to extend and validate the use of the rat olfactory sensory neurons as models of the

DOI: 10.1021/cn900042c | *ACS Chem. Neurosci.* (2010), *1*, 246–256

human system. Because human (*11*) and rat (*12*) olfactory neurons respond to odorants with increases in cytoplasmic calcium, we used ratiometric calcium imaging and electrophysiological methods to examine responses to nicotine in olfactory neurons. Our results show that the odor of nicotine is not due to activation of nicotinic acetylcholine receptors but rather due to the activation of a canonical olfactory pathway. This interpretation is supported by the finding that the response to nicotine in olfactory sensory neurons (OSNs) is dependent on the classical cAMP-dependent second messenger pathway and apparently involves activation of at least three types of odorant receptor neurons with distinct response profiles to the enantiomers of nicotine.

Results

S(−)-Nicotine Induces Increases in Intracellular Calcium, $[Ca^{2+}]_i$, in OSNs

Freshly isolated rat OSNs respond to nicotine with several patterns of change in $[Ca^{2+}]_i$. In response to a series of increasing concentrations of *S*(−)-nicotine, the response magnitude of some individual neurons increased monotonically, without sensitization or desensitization, across the range of tested concentrations (Figure 1A, n = 5 of 6480 KCl-sensitive cells tested). More commonly, cells that were sensitive to nicotine responded to at least one concentration in the range of 30−3000 μM and then desensitized to subsequent stimuli in the series (n = 69 of 6480 KCl-sensitive cells tested, Figure 1B). Thus, the threshold of response ranged from 30 to 3000 μM. Concentration dependence can also be expressed as the percent of neurons responding to a given concentration (Figure 2). In other experiments in which neurons were presented with multiple stimulations by a single concentration of *S*(−)-nicotine, sensitization was observed. These neurons failed to respond to the initial few stimulations but ultimately did respond to nicotine (Figure 1C, n = 31 of 6480 KCl-sensitive cells tested).

Cultured human olfactory cells also responded to *S*(−)-nicotine in a concentration-dependent manner (Figure 1D).

Freshly biopsied human OSNs responded to 1 mM *S*(−)-nicotine with increases in $[Ca^{2+}]_i$ (68% of 81 morphologically identified neurons) but an insufficient number of nicotine responsive cells were obtained to enable a complete determination of a concentration/response function.

Enantiomeric Selectivity for *S*(−)- and *R*(+)-Nicotine

We observed enantiomeric selectivity in some OSNs, while others were nonselective. Selective neurons were scored as such only if the lack of response to one enantiomer occurred to the first stimulus. By this criterion,

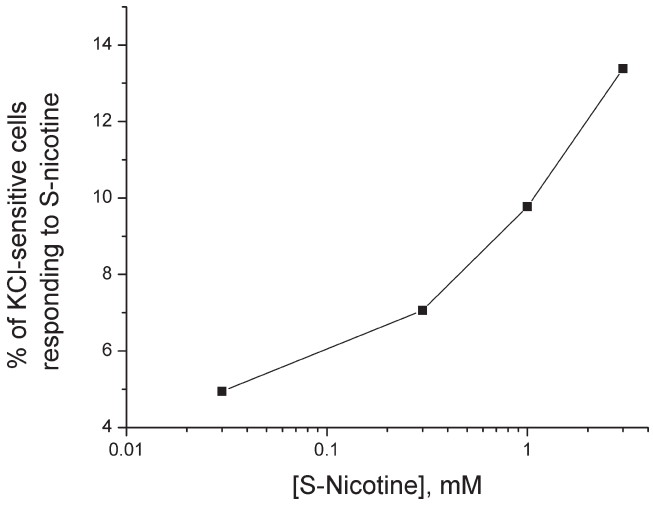


Figure 2. Rat olfactory sensory neurons exhibited a wide range of response thresholds when stimulated with a series of increasing concentrations of *S*(−)-nicotine. The figure plots the percentage of neurons that exhibited their first suprathreshold response at the indicated concentration.

selectivity cannot be attributed to adaptation or desensitization. In rat OSNs, the frequency of cells that were selectively responsive to *S*(−)-nicotine was similar to the frequency of nonselective cells (Table 1, Figure 3A), with more cells in total responding to *S*(−)-nicotine, the naturally occurring enantiomer. In contrast, in freshly dissociated human cells (Figure 3B) and cultured human olfactory cells (Figure 3C), the majority of responsive cells were responsive to both enantiomers, and only a few enantiomerically selective cells were observed (Table 1).

The Increase in $[Ca^{2+}]_i$ in Response to Nicotine Depends on Extracellular Calcium

Removal of extracellular calcium inhibited the response of rat OSNs to *S*(−)-nicotine by 96.5% ± 1.3% (mean ± SE, n = 17, Figure 4A), indicating that the changes in $[Ca^{2+}]_i$ induced by nicotine were dependent on an influx of calcium. Frequently, when the extracellular calcium was returned to the bath solution, rat OSNs displayed an increase in $[Ca^{2+}]_i$ (Figure 4A), possibly due to residual nicotine or to ion channels remaining open following the exposure to nicotine. In all cultured human cells tested, the nicotine-induced increase in $[Ca^{2+}]_i$ was abolished by the removal of extracellular calcium (n = 21, Figure 4B).

The Increase in $[Ca^{2+}]_i$ Caused by Nicotine Stimulation of OSNs Is Not Mediated by nAChRs

Neither the magnitude nor the latency (Figure 5) of the response to *S*(−)-nicotine was affected by inhibitors of the nAChR at commonly used concentrations. The responses of rat OSNs to *S*(−)-nicotine in the presence of the noncompetitive inhibitor mecamylamine were

DOI: 10.1021/cn900042c | *ACS Chem. Neurosci.* (2010), *1*, 246–256

Table 1. Frequency of Responses (Measured Using Calcium Imaging) to 1 mM *S*(−)-Nicotine Only, 1 mM *R*(+)-Nicotine Only, and Both Enantiomers (Each 1 mM) in Rat OSNs and Human Cultured Olfactory Cells

	total OSNs tested	OSNs responding to *S*(−)-nicotine only	OSNs responding to *R*(+)-nicotine only	OSNs responding to both
rat, 1 mM	2386 (1938)[a]	28 (1.2%) 26 (1.3%)[a]	10 (0.4%) 8 (0.4%)[a]	30 (1.3%) 28 (1.4%)[a]
human cultured cells, 1 mM	1393	6 (0.4%)	4 (0.2)	16 (1.2%)

[a] Nicotine at 3 mM.

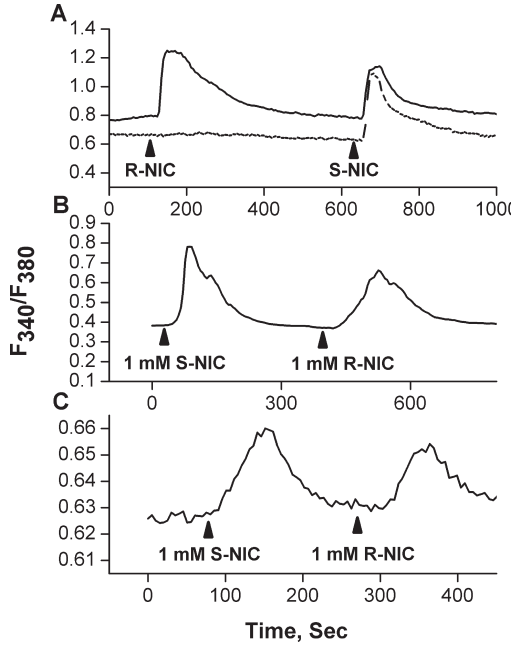


Figure 3. Nicotine-sensitive cells responded to either single or both enantiomers. In rat OSNs, cells responsive to only *S*(−)-nicotine (dotted line) and nonselective cells (solid line) were observed at similar frequency (A). Many nicotine-sensitive freshly biopsied human OSNs were not stereoselective; 61.5% responded to both enantiomers (B). Similarly, 96.6% of cultured nicotine-sensitive human olfactory cells were not stereoselective (C).

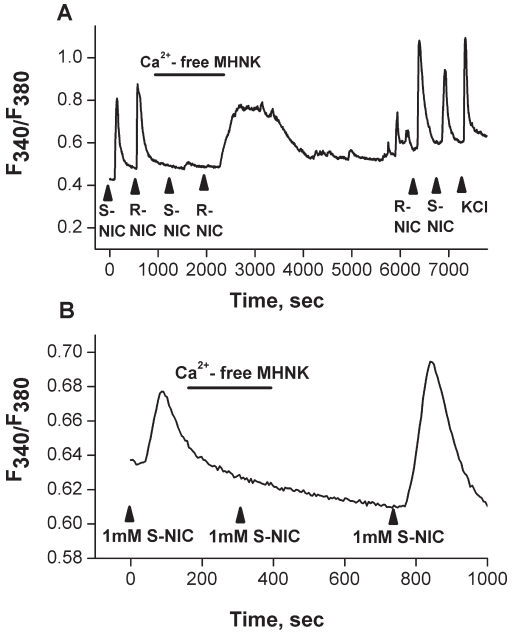


Figure 4. OSN responses to *S*(−)- and *R*(+)-nicotine depended on the presence of extracellular calcium. Removal of extracellular calcium strongly inhibited responses to *R*(+)- and *S*(−)-nicotine in rat OSNs (n = 17, A) and completely abolished responses to *S*(−)-nicotine in human cultured olfactory cells (n = 21, B).

within 96% of the response before and after drug washout (n = 25), while those of OSNs exposed to the competitive nicotinic antagonist hexamethonium were within 98% of the corresponding untreated values (n = 3, Figure 5A,B). The responses of cultured human olfactory cells were similarly unaffected by either mecamylamine (n = 72) or hexamethonium (n = 70) (Figure 5C,D).

One freshly dissociated human OSN responding to only *R*(+)-nicotine was also unaffected by mecamylamine (Figure 5E). We also used a nicotinic acetylcholine receptor agonist, dimethylphenylpiperazinium (DMPP) to further demonstrate that responses to *S*(−)-nicotine are not mediated by classical nicotinic receptors and that these nicotinic receptors appear not to be present on OSNs responsive to *S*(−)-nicotine. DMPP did not stimulate *S*(−)-nicotine-sensitive rat OSNs (n = 10, Figure 5F).

Nicotine Elicits Calcium Influx via cAMP Second Messenger Pathways

S(−)-Nicotine acts on OSNs mainly via the adenylate cyclase pathway. Neither neomycin, an antagonist of phospholipase C (PLC, 1 mM) (*19*), nor thapsigargin, a sarcoplasmic reticulum Ca^{2+}-ATPase inhibitor (200 nM) (*20*), altered the response to nicotine (1 mM) in rat OSNs (Figure 6A) in 16 of 17 rat OSNs tested. Another antagonist of PLC, U73122, had little or no effect on the response of most cells to *S*(−)-nicotine. The mean magnitude of the response of rat OSNs (16 of 18 tested) to *S*-nicotine during exposure to U73122 was 82.7% ± 18.0% of the control response (Figure 6B). Two of the 18 nicotine-sensitive OSNs tested were completely inhibited by U73122. In 7 of the 10 U73122-insensitive cells (inhibited less than 20% of control) that were tested, the nicotine response was completely abolished by MDL12,330A (20 μM), an inhibitor of adenylate cyclase (*21*) (Figure 6B). In two rat OSNs, responses to

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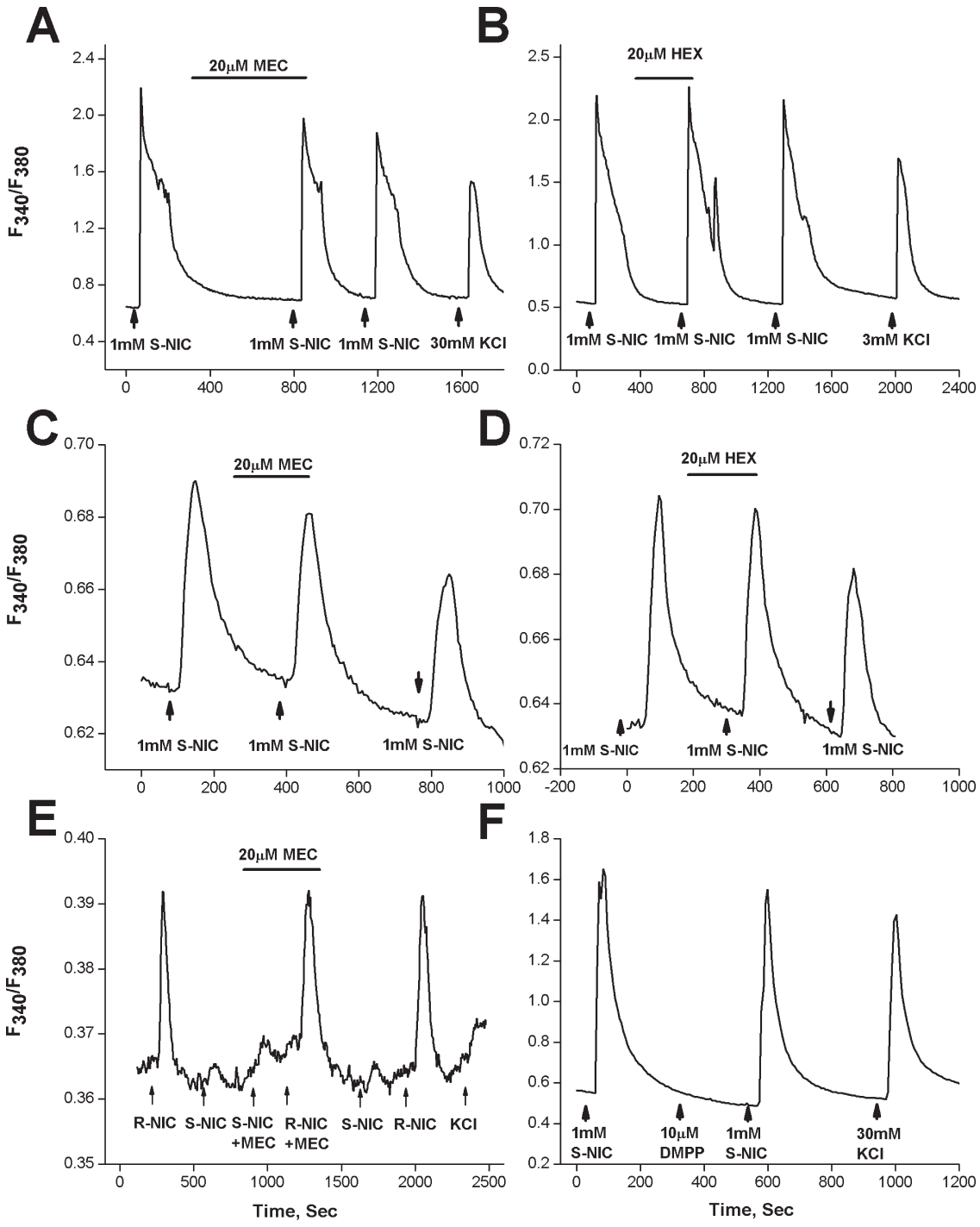

Figure 5. Neither 20 μM mecamylamine nor 20 μM hexamethonium had an effect on responses to 3 mM *S*(−)-nicotine in rat OSNs (A,B) and human cultured olfactory cells (C,D). Mecamylamine also had no effect on nicotine responses in fresh human biopsy OSNs (E). DMPP (10 μM) did not stimulate nicotine-sensitive rat OSNs (F).

S-nicotine were unaffected by either U73122 or MDL-12,330A.

Characterization of Nicotine-Induced Currents in Rat Olfactory Neurons

To examine the current characteristics underlying the calcium response of OSNs to nicotine, we used perforated-patch electrophysiology. Figure 7A shows an example of the nicotine-induced current, which was inward at negative holding potentials and outward at positive ones. The current−voltage relationship of the induced current (Figure 7B) suggests the activation of a non-specific cation current with an almost linear slope and a

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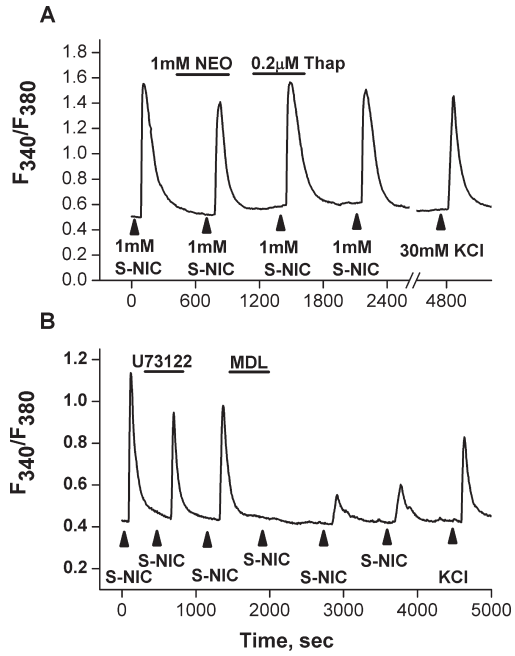

Figure 6. *S*(−)-Nicotine acts on OSNs via the adenylate cyclase pathway. Neither neomycin (1 mM) nor thapsigargin (200 nM) altered the response to nicotine (3 mM) in rat OSNs (A). Although U73122 had little or no effect on the response of most cells (~17% inhibition) to *S*(−)-nicotine, the nicotine response was completely antagonized by MDL12,330A (20 μM), an inhibitor of adenylate cyclase (B).

reversal potential around 0 mV. We did not specifically test for the presence of a calcium-activated chloride conductance, which has been reported to also reverse around 0 mV (*22*). A similar current was observed by us and others in response to elevation of cyclic AMP by forskolin or isobutylmethylxanthine (IBMX; data not shown (*23*)). To determine whether high calcium would inhibit the conductance, as expected for the cyclic nucleotide gated channel (*24*), we applied 3 mM nicotine in the presence of 1 mM Ca^{2+} in the bath or in nominally Ca^{2+}-free solution while measuring the membrane potential under current-clamp conditions. Figure 7C shows that the nicotine-induced depolarization, in the presence of Ca^{2+}, was transient and ended before the termination of the stimulus. An action potential was generated during the depolarizing phase of the response. In Ca^{2+}-free solution, the depolarization was larger and sustained, and the membrane potential returned to baseline only after wash-out of the stimulus. These data demonstrate the Ca^{2+}-sensitivity of the nicotine-induced current (Figure 7D), consistent with activation of the cyclic nucleotide gated channel.

Stimulation with *S*(−)-nicotine (3 mM) elicited nonspecific cation currents or led to depolarization in 7 out of 17 rat OSNs. Of the observed depolarizing responses, four (24%) were due to putative cation currents, while three (18%) were due to outward current suppression.

Discussion

The flavor of nicotine in tobacco smoke is comprised of olfactory, trigeminal, and gustatory components. The current studies examined the detection mechanisms underlying the olfactory component of nicotine flavor and compared rat and human olfactory cell response characteristics.

Nicotine Stimulates Olfactory Receptors That Are Not nAChRs

The odor of nicotine is described as sweet, warm, or spicy at low to moderate concentrations (*8*). Electro-olfactograms (EOGs) have been measured from the olfactory sensory epithelium in rat in response to nicotine vapor (*25*) but were not detected from nasal respiratory epithelium. This suggests that the potential measured was not due to somatosensory nerve endings but rather to olfactory receptors. In humans, these receptors do not appear to be nicotinic acetylcholine receptors (nAChRs) because neither detection thresholds nor intensity ratings for the odor of nicotine were affected by the nicotinic AChR antagonist mecamylamine (*10*). The lack of effect of mecamylamine suggested that either (1) the drug did not have access to nAChRs coupled to olfactory responses or (2) the odor of nicotine is mediated via canonical olfactory receptors, the binding sites of which are distinct from nAChRs. Our data not only suggest that is the odor of nicotine mediated by olfactory receptors but also show that nAChRs are not active on olfactory receptor neurons. Human and rat olfactory neurons, as well as cultured human olfactory cells, responded to nicotine with increases in intracellular calcium. Several additional lines of evidence indicate that this response was mediated by olfactory receptors instead of nicotinic acetylcholine receptors. First, both the competitive antagonist hexamethonium and the noncompetitive antagonist mecamylamine failed to inhibit OSN responses to *S*(−)-nicotine. Second, neurons that were responsive to *S*(−)-nicotine failed to respond to DMPP, a general nicotinic receptor agonist.

Humans can detect and discriminate the *S*(−) and *R*(+) enantiomers of nicotine, and both smokers and nonsmokers rate the *S*(−) enantiomer "more pleasant" than the *R*(+) enantiomer (*26*). Moreover, subjects assigned different qualitative descriptors to *S*(−)- and *R*(+)-nicotine (*8*). This suggests the existence of at least two populations of nicotine-sensitive olfactory receptors with sufficient stereoselectivity to support this fine discrimination. Consistent with this, we found that there were enantiomerically specific responses to *S*(−)- and *R*(+)-nicotine, with two populations of cells each responding solely to one enantiomer or the other and a third population that

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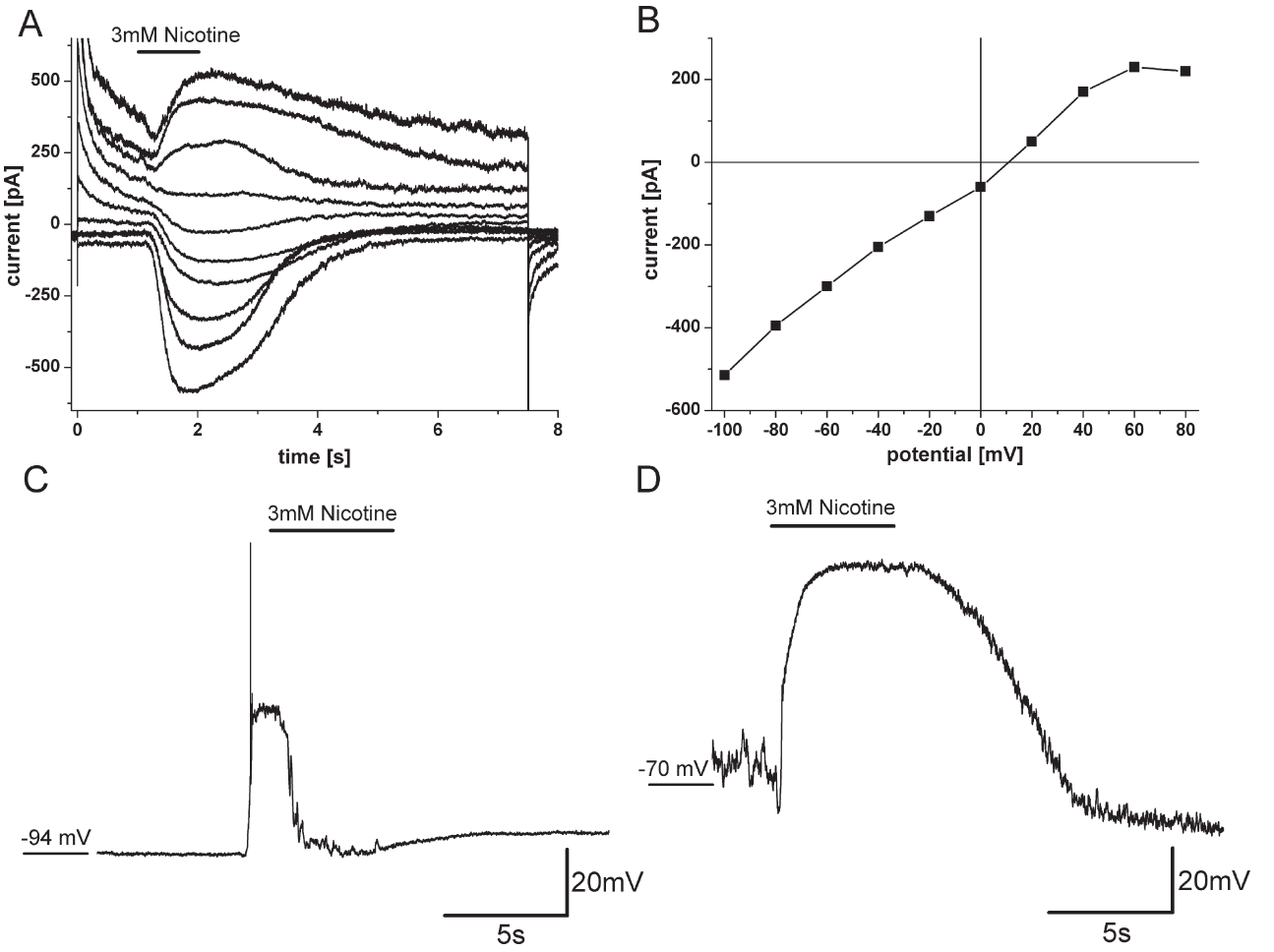

Figure 7. Whole cell currents obtained from an isolated rat olfactory neuron in response to 3 mM *S*(−)-nicotine (A). *I*−*V* relationship for 3 mM *S*(−)-nicotine (B). Bath calcium concentration in panels A and B is 1 mM. In the presence of extracellular Ca^{2+} (1 mM), nicotine currents desensitized rapidly (C), while in the absence (no added Ca^{2+}, no EGTA) of extracellular Ca^{2+}, the repolarization occurred only after the removal of nicotine (D). Panels C and D are from the same neuron.

responded to both enantiomers. Assuming expression of only one receptor type per neuron (*27*), these results indicate that there are a minimum of three different types of nicotine-sensitive olfactory receptor cells in the populations of OSNs that we studied. Compared with rat OSNs, there were relatively fewer human sensory cells that were enantiomerically selective. The discrimination of nicotine enantiomers that has been reported (*25*) may be mediated by the small proportion of cells that we found or by a greater number of selective sensory neurons that might be found in areas of the olfactory sensory epithelium that we could not sample in humans.

While the demonstration of enantiomeric selectivity supports multiple nicotine-responsive olfactory receptors, these data alone do not exclude the possibility that nAChRs may be present on other cells within the OE. A number of nAChR subunits have been identified by PCR from human middle turbinate tissue (*28*) and rat olfactory epithelium (*29*). Subunits α3, α7, and β2 were found in all of the human middle turbinate specimens studied, and α5, α9, and β2 were found in rat OE, although different combinations of subunits were detected in each specimen and gender-associated differences were suggested. Binding studies have also demonstrated nicotinic binding sites in the rat olfactory epithelium but have not identified the cell types responsible for this binding (*29*, *30*). The modulatory effects of acetylcholine on frog OSNs can be antagonized by tubocurarine, a competitive nAChR antagonist, suggesting that frog OSNs may express this receptor, although nonspecific effects on nicotine-sensitive olfactory receptors cannot be ruled out (*31*). Our results indicate that since olfactory neuronal responses to nicotine were not mediated by nAChRs, the nAChRs found in human turbinate tissue (*28*) and rat olfactory epithelium (*29*) are more likely associated with non-OSN cells such as supporting, basal, or secretory cells, trigeminal nerve endings, or other underlying tissue.

DOI: 10.1021/cn900042c | *ACS Chem. Neurosci.* (2010), *1*, 246–256

Nicotine Responses Are Mediated by Adenylate Cyclase and Cyclic Nucleotide Gated Channels

Several lines of evidence suggest that nicotine-activated responses of olfactory neurons are due to activation of a cAMP-dependent cation influx, a hallmark of the primary olfactory signal transduction pathway (*32*). Electrophysiological data identified the channel as a nonspecific cation channel. Both the cyclic nucleotide gated channels of OSNs and nicotinic ACh channels are nonspecific cation channels. However, the cyclic nucleotide gated channel is inhibited by high $[Ca^{2+}]_i$, which can be achieved using high extracellular calcium (*23*, *33*), while the nicotinic ACh channels contain a larger pore and are easily permeable to a number of cations and are not inhibited by calcium (*34*). The nicotine-induced conductance was reduced by the presence of extracellular Ca^{2+}, which together with the observed antagonism of the nicotine response by MDL12,330A, an inhibitor of adenylate cyclase (*21*), supports the notion that nicotine activated cyclic nucleotide gated channels via cAMP as the second messenger. Interestingly, nicotine activation of the cyclic nucleotide gated channels in the presence of high extracellular calcium did not enhance the response in a manner consistent with the activation of calcium-gated chloride channels. The possibility that nicotine may interfere with calcium-activated chloride channels could explain the absence of such currents.

S-Nicotine induced current responses in a higher proportion of cells than increases in intracellular calcium. However, there are several observations that bring these two results more into agreement. Odorant suppression of an outward current has been previously observed in rat olfactory neurons (*18*) and a suppression of slowly inactivating potassium currents in cultured neurons by nicotine has also been descibed (*35*). While outward current suppression was also obseved in the set of OSNs tested here and counted as responses, it would not, under resting conditions, lead to an increase of intracellular calcium. Depolarizing responses based on activation of the canonical cAMP pathway also occurred at a somewhat higher frequency than the NIC-induced calcium responses. One explanation may simply be that responses that are clearly identifiable electrophysiologically could be subthreshold for the opening of voltage-gated calcium channels and only elicit small, hard to detect calcium influxes through cyclic nucleotide gated channels. Electrophysiological meassurements also demand higher viability of the recorded cells and thus bias the response frequencies using this method.

Conclusion

The olfactory sensation of nicotine depends on the peripheral activation of noncholinergic receptors in the olfactory system and activation of components of the canonical olfactory pathway. Since the odor of nicotine may become a conditioned stimulus that further reinforces and supports smoking behavior, a better understanding of the contribution of the olfactory sensitivity to nicotine, as well as to other components of tobacco smoke, should aid in the development of novel, improved smoking cessation strategies.

Experimental Methods

Materials

Mammalian Ringer's solution (Ringer's) contained (in mM): 145 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 1 sodium pyruvate, 20 *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES, pH 7.2). For Ca^{2+}-free Ringer's, $CaCl_2$ was removed and replaced with 1 mM ethylene glycol tetraacetic acid (EGTA). Isolation solution contains (in mM): 145 NaCl, 5 KCl, 2 EDTA, 1 sodium pyruvate, 20 HEPES, 100 μg/mL gentamycin. Stop solution contains (in mM): 145 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 5 D-glucose, 1 sodium pyruvate, 20 HEPES. Cell culture medium consisted of Iscove's Dulbecco's modified Eagles medium (IDMEM high glucose) supplemented with 10% fetal bovine serum (heat inactivated) and 1% penicillin/streptomycin (100 IU and 100 μg/mL, respectively).

Other compounds used in this study were as follows: *S*(−)-nicotine (Philip Morris, Richmond, VA, >99% by GC), *R*(+)-nicotine (Toronto Research Chemical Inc., North York, Canada, 97% by TLC, NMR and optical rotation), noncompetitive nicotinic acetylcholine receptor antagonist mecamylamine hydrochloride (20 μM), competitive nicotinic acetylcholine receptor antagonist hexamethonium chloride (20 μM), nicotinic acetylcholine receptor agonist dimethylphenylpiperazinium (DMPP, 10 μM), adenylate cyclase inhibitor MDL-12,330A hydrochloride (20 μM), phospholipase C antagonists U-73122 (20 μM) and neomycin (1 mM), sarcoplasmic reticulum Ca^{2+}-ATPase inhibitor thapsigargin (200 nM, Calbiochem, San Diego, CA), adenylate cyclase activator forskolin, phosphodiesterase inhibitor isobutylmethylxanthine (IBMX), leupeptin, L-cysteine, papain, and DNase I (Roche Diagnostics, Indianapolis, IN). Unless otherwise specified, all compounds were from Sigma-Aldrich, Inc. (St Louis, MO).

Human Subjects and Biopsy of Olfactory Tissue

Work with human tissue described in this manuscript was conducted in accordance with the Declaration of Helsinki using procedures approved by the Institutional Review Board of Thomas Jefferson University, Philadelphia, PA. Subjects provided informed consent by signing a document describing the nature and possible consequences of participation. The subjects were five men and seven women, ages 23−61 (28.3 ± 11.8, mean ± SD), two of whom smoked and 10 who had never smoked. All subjects performed within normal limits on unilateral detection threshold testing for phenylethylalcohol using standard procedures (*13*), did not report any current sinus problems or taste or smell disorders, and were not taking any medications that would exclude them from participation (anticoagulants or sinus or allergy medications). An ∼1 mm^3 tissue sample was excised from the middle turbinate and opposing septum under local anesthesia using established olfactory

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biopsy techniques (*14*, *15*). Biopsies were placed immediately into IDMEM and transported to the laboratory for further processing.

Animal Tissues

The use and handling of animals was performed in accordance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals and the National Institutes of Health guide (NIH Publications 80-23, revised 1978). The experimental protocols were approved by the Institutional Animal Care and Use Committee of Monell Chemical Senses Center, and all efforts were made to reduce the number of animals used and minimize animal suffering. Animals were euthanized with CO_2, and the turbinates and septum (from 150 to 250 g, 50−75 day old Sprague−Dawley rats) were immediately excised and transferred into isolation solution.

Preparation of Dissociated Olfactory Sensory Neurons and Primary Cell Culture

For the acute preparation of both human and rat OSNs, the tissue was finely minced and incubated in isolation solution with 5 μL/mL papain and 5 mM L-cysteine for 15 min. Following cell dissociation, the tissue was gently triturated with a fire polished pipet, transferred into a stop solution containing 10 μg/mL leupeptin and 20 U of DNase I and filtered with a 70 μm nylon cell strainer (BD Falcon, Bedford, MA). The dissociated cells were layered on top of a 20%/40% Percoll gradient and centrifuged for 10 min at 700*g*. The olfactory cells were harvested from the 20%−40% interface.

Primary cultures of human olfactory epithelial cells for both calcium imaging and electrophysiology experiments were prepared as described previously (*2*). Briefly, biopsies were minced coarsely and incubated in the calcium- and magnesium-free HBSS for ∼20 min, triturated, and centrifuged at 600*g* for 5 min. The pellet was resuspended in culture medium and transferred to a 25 cm^2 culture flask and allowed to grow for 1−2 weeks until cell growth was sufficient for transfer to continuous culture. Cells were maintained in 75 cm^2 culture flasks in a humidified incubator (37 °C, 5% CO_2). Cultured cells established in this fashion grew to confluence in 1 week. Based on previous experiments an optimum number of mature neurons (based on assays of odorant responsiveness or immunocytochemical detection of molecular markers such as olfactory marker protein (*2*, *16*) were present by day 4−5 after plating. Cultures could be frozen at passage 2−3, thawed, and used to generate new cultures with similar growth rates and proportions of mature cells, and typically became senescent after 6−10 passages. The current protocol is identical to earlier studies (*2*) in which approximately 65% of subject biopsies generated successful cultures based on proliferation and generation of cells showing calcium responses to odorants.

Measurement of Cellular Responses to Stimulation

Cellular responses were measured using ratiometric calcium imaging techniques as previously described (*17*). Rat and freshly biopsied human OSNs were loaded with 5 μM acetoxymethylester of Fura-2 (Fura-2/AM) and 80 μg/mL pluronic F127 (Molecular Probes, Eugene, OR) for at least 1 h at room temperature and transferred onto coverslips (22 × 60 mm^2, No. 0, Thomas Scientific Co.) coated with 1 mg/mL concanavalin A. Cultured human olfactory cells (HOCs) were similarly loaded with Fura-2/AM after having been plated onto glass coverslips and cultured for 4−5 days. Coverslips with OSNs or HOCs were set in a recording chamber and superfused with Ringer's solution or Ringer's containing different chemical compounds via a valve controller (VC-8, Warner Instruments, Hamden, Connecticut) and perfusion pump (Perimax 12, SPETEC, Erding, Germany). Stimulation duration was 30 s, and perfusion rate was 0.8 mL/min. There was a 20 s delay between solenoid activation and arrival of stimulus compounds at the neurons. The Photon Technology International (PTI) calcium imaging system was as follows: excitation from a DeltaRAM X monochromator was at 340 nm and 380 nm with a 510 nm emission filter. Cellular fluorescence was imaged (20×, Olympus IX70) with a Cooke CCD camera. Images were digitized and analyzed using Imagemaster (PTI) software. Since there were multiple types of cells in the dissociated tissue preparation, rat and human olfactory neurons were identified morphologically, and only cells with rounded cell bodies and a dendrite with knob (Figure 1A, inset) were considered to be olfactory sensory neurons. The fluorescence ratio (F_{340}/F_{380}) was calculated for regions-of-interest drawn around these cells. Cells were considered responsive if they displayed a 10% increase in intracellular calcium.

Electrophysiology

Perforated patch electrophysiology was carried out with freshly dissociated rat olfactory cells according to previously published protocols (*18*). Olfactory neurons were identified morphologically, and only cells with rounded cell bodies, a dendrite with knob, and cilia were selected for recording. An Axopatch 200A amplifier (Axon Instruments) and a PC running PClamp 8 were used to apply pulse protocols and record data. Cells were recorded in perforated-patch configuration using gramicidin (120 μg/mL) as ionophore. Patch pipettes were fabricated from borosilicate glass (Kimex 51, Kimble Glass, Inc.) on a vertical pipet puller (PP-83, Narishige) and fire polished to a final bath resistance of 3−5 MΩ. After formation of a giga seal, cell membranes generally began to perforate within 5 min, and steady-state access was reached after ∼15 min, at which point large voltage-activated sodium and potassium currents could be elicited by depolarizing voltage steps. Stimuli were applied via a multibarrel pipet connected to a Picospritzer unit. Cells were continuously rinsed with Ringer's after mounting on the microscope. Prior to stimulation, the bath solution (initially 1 mM Ca^{2+} Ringer's) was changed to 0 Ca^{2+} Ringer's (nominally calcium-free). The pipet solution contained (in mM): 110 potassium aspartate, 36 KCl, 1 $MgCl_2$, 1 EGTA, 10 HEPES, 1 ATP, 0.1 GTP. The pH was adjusted to 7.2 with KOH, and the osmolarity was 305 mOsmol.

Author Contributions

Drs. Bryant and Rawson oversaw experimental design and manuscript preparation. Dr. Jiang designed and performed calcium imaging experiments with assistance from V. Audige who prepared human and animal cells. Dr. Lischka designed and performed all electrophysiological experiments.

⊥ These authors contributed equally to this research.

Funding Sources

Funding was provided by Philip Morris USA and the Monell Chemical Senses Center.

DOI: 10.1021/cn900042c | *ACS Chem. Neurosci.* (2010), *1*, 246–256

Notes

We disclose no conflicting financial or other interests.

Acknowledgment

We thank Dr. E. Pribitkin for providing olfactory biopsy samples, C. Klock and A. Vainius for subject recruitment and testing, and R. Peoples, G. Shaffer, and C. Luongo for technical, computer, and programming support. We also acknowledge G. Kobal, P. Wise, J. Mennella, and Y. Pepino for manuscript review and discussion.

Abbreviations

ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; DMPP, 1,1-dimethylphenyl 1,4-piperazinium; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol tetraacetic acid; EOG, electroolfactogram; GTP, guanosine triphosphate; HBSS, Hanks buffered saline solution; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IBMX, isobutylmethylxanthine; IDMEM, Iscove's modification of Dulbecco's modified Eagles medium; nAChR, nicotinic acetylcholine receptor; NMP, nasal mucosal potential; OE, olfactory epithelium; OSN, olfactory sensory neuron; PCR, polymerase chain reaction; ROI, region of interest.

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