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Potential of evoked calcitonin gene-related peptide release from oral mucosa: a potential basis for the pro-inflammatory effects of nicotine

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Abstract

Inflammation of the buccal mucosa, gingiva and periodontal tissues is a significant problem in users of nicotine-containing tobacco products; however, the potential role of nicotine in the development of this inflammation is unclear. In many tissues, nicotine, acting through nicotinic acetylcholine receptors (nAChRs), has been shown to increase the release of the pro-inflammatory mediator calcitonin gene-related peptide (CGRP) thereby potentially contributing to neurogenic inflammation. The purpose of the present studies was to determine the effects of nicotine and other nAChR agonists on capsaicin-evoked immunoreactive CGRP (iCGRP) release from rat buccal mucosa and to identify a potential cellular basis for these effects. Using a previously validated model of *in vitro* superfusion, we show that the nAChR agonists nicotine (EC₅₀ 557 μ M), epibatidine (EC₅₀ 317 pM) and cytisine (EC₅₀ 4.83 nM) potentiated capsaicin-evoked iCGRP release in a concentration-dependent manner by 123, 70 and 76%, respectively. The expression and distribution patterns of the mRNA transcripts encoding the α 3, α 4 and α 6 nAChR subunits and their colocalization with CGRP and the capsaicin receptor VR1 were examined in rat trigeminal ganglion using combined *in situ* hybridization and immunohistofluorescence. Of all trigeminal neurons counted, mRNA encoding the α 3, α 4 and α 6 subunits was found, respectively, in 14.45, 9.2 and 19.21% of neurons. The cell body diameter of most neurons containing any nAChR subunit was in the 30–40 μ m range with slightly fewer in the 20–30 μ m range. Co-localization of these α subunit transcripts with either CGRP or VR1 immunoreactivity ranged from approximately 5 to 7% for α 4 and over 8% for α 3 to 18% for α 6. These data support the hypothesis that nicotinic agents, acting at nAChRs contained on primary sensory neurons, are capable of directly modulating the stimulated release of iCGRP. In the case of users of nicotine-containing tobacco products, this modulation could contribute to inflammatory processes within the oral cavity.

Keywords

buccal mucosa; capsaicin; cytisine; epibatidine; nicotinic acetylcholine receptor; sensory neuron

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Introduction

Neurogenic inflammation is a process that occurs in response to the stimulation of certain peripheral primary sensory neurons (Jancsó *et al.*, 1967). This process is mediated in part by several pro-inflammatory neuropeptides, including calcitonin gene-related peptide (CGRP) and substance P, that are released from the peripheral terminals of these neurons (Gazeliu *et al.*, 1987; Cruwys *et al.*, 1992; Karimian & Ferrell, 1994; Brain *et al.*, 1985; Brain & Williams, 1985). In support of the involvement of these neuropeptides in the inflammatory response, it has been shown that CGRP and substance P undergo axonal transport from the nodose and dorsal root ganglia to the periphery along the vagus and sciatic nerves, respectively (Brimijoin *et al.*, 1980; Kashihara *et al.*, 1989), and that their peripheral administration produces vasodilation (Brain *et al.*, 1985; Gazeliu *et al.*, 1987) and plasma extravasation (Gamse & Saria, 1985). In addition, antidromic electrical stimulation of the trigeminal ganglion causes vasodilation in facial skin through a process that is dependent on CGRP (Escott *et al.*, 1995). Collectively, these studies strongly implicate CGRP as a mediator of neurogenic inflammation and validate its utility as a marker for this process in a variety of experimental settings.

Nicotinic acetylcholine receptors (nAChRs) are members of the ligand-gated ion channel superfamily. The pentameric stoichiometry of these receptors comprises two alpha subunits and three beta subunits (Anand *et al.*, 1991; Cooper *et al.*, 1991) or, in the case of α -bungarotoxin-sensitive nicotinic receptors, five alpha subunits. Activation of these receptors results in a conformational change in the receptor complex, allowing the conductance of Na^+ , K^+ and Ca^{2+} ions to varying extents depending on the nAChR subtype(s) involved. Subunits known to be expressed in the mammalian nervous system include $\alpha 2$ – $\alpha 7$, $\alpha 9$, $\alpha 10$ and $\beta 2$ – $\beta 4$. In the rat trigeminal ganglion, subtypes made up of $\alpha 4\beta 2$ and $\alpha 3\beta 4$ subunit combinations have been demonstrated as has mRNA encoding the $\alpha 2$, $\alpha 5$ – $\alpha 7$, $\alpha 9$ and $\beta 3$ subunits (Wada *et al.*, 1989, 1990; Flores *et al.*, 1996; Liu *et al.*, 1998; Keiger & Walker, 2000). However, the functional role of these sensory neuronal nAChRs is not well understood.

Nicotine is capable of modulating the activity of sensory neurons and the transmitter substances they secrete. Nicotine or other nicotinic agonists applied to sensory neurons has been shown to activate these neurons both *in vitro* (Steen & Reeh, 1993; Liu & Simon, 1993) and *in vivo* (Tanelian, 1991). Nicotine is known to directly stimulate the release of immunoreactive CGRP (iCGRP) in several tissues including heart (Franco-Cereceda *et al.*, 1991, 1992), trachea (Hua *et al.*, 1994; Jinno *et al.*, 1994), pulmonary tissue (Lou *et al.*, 1991, 1992) and cultured dorsal root ganglion neurons (Franco-Cereceda *et al.*, 1992). In addition, nicotine enhances the evoked release of iCGRP in dental pulp (Hargreaves *et al.*, 1992) and rat paw skin (Kilo *et al.*, 1995). Taken together, these studies show that nicotine is capable of modulating the release of CGRP from sensory neurons and may thereby contribute to the process of neurogenic inflammation.

Similar to studies on the trunk and limbs, evidence shows that the development of certain oral inflammatory diseases has a neurogenic component (Györfi *et al.*, 1992). The link between the use of nicotine-containing tobacco products and increases in the incidence of oral inflammatory disease is well established. For example, smoking is associated with an increase in periodontal disease (Haber *et al.*, 1993) and gingivitis (Ismail *et al.*, 1983). The studies detailed here sought to provide a potential mechanistic explanation linking nicotine exposure of oral tissue to inflammatory disease. To address this hypothesis, studies were performed using nAChR agonists in a previously validated model system for the study of neurogenic inflammation in oral tissues (Flores *et al.*, 2001). In addition, nAChR subunits

were localized to CGRP-/VR1-containing neurons using combined *in situ* hybridization and immunohistofluorescence.

Materials and methods

Animals

All experiments were carried out in accordance with protocols approved by the Institutional Animal Care and Use Committee of The University of Texas Health Science Center at San Antonio as well as with the guidelines for the ethical treatment of animals established by the National Institutes of Health. All experiments utilized tissues obtained from adult (175–250 g), male Sprague-Dawley rats (Charles River, Wilmington, MA, USA).

In vitro superfusion

Rats were killed by decapitation and the buccal mucosae were dissected from the underlying buccinator muscle and placed in a superfusion chamber (the mucosae from both sides of the oral cavity of one animal per chamber) housed in a 37 °C waterbath (for details see Flores *et al.*, 2001). The tissue was continuously supervised with Krebs buffer containing 1 mM NaH₂PO₄, 135 mM NaCl, 3.5 mM KCl, 1 mM NaHCO₃, 1 mM MgCl₂, 2.5 mM CaCl₂, 0.1 mM ascorbic acid, 16 µM thiorphan (BACHEM, Bubendorf, Switzerland), 3.3 mM dextrose, 10 mM Hepes and 0.1% bovine serum albumin at a flow rate of approximately 0.21 mL/min. The buffer (pH 7.4) was oxygenated and immersed in a separate 37 °C waterbath. Superfusate was collected in 12 fractions, 10 min each, using an automated fraction collector (Gilson, Middleton, WI, USA). Tissue was perfused for 70 min, followed by a 10-min application of buffer containing either capsaicin or capsaicin plus drug. To maximize the opportunity of observing nicotinic agonist-induced facilitatory or inhibitory effects, whichever occurred, in all superfusion experiments, capsaicin was used at a submaximal approximately EC₈₀ concentration (100 µM) as previously described. Cytisine (Sigma, St. Louis, MO, USA), (–)-nicotine hydrogen tartrate salt (Sigma) and epibatidine (RBI, Natick, MA, USA) were dissolved in distilled H₂O and diluted in Krebs buffer. The length of tubing between the reservoir and chamber was designed so that there would be a 3-min lag period between the time the drug was added and the time it would reach the chamber. Thus, drug treatments were applied 3 min before the start of the next fraction. Varying concentrations of drug in combination with 100 µM capsaicin (Fluka, Ronkonkoma, NY, USA) were added to individual reservoirs of buffer to determine concentration–response curves for each drug. Each concentration was applied to one group of chambers (*n* = 5–6) and compared with chambers receiving buffer containing capsaicin only (*n* = 6). Tissue was stimulated only once and superfusion was completed with a 40-min washout period using Krebs buffer alone. In antagonist experiments, buffer containing mecamylamine (Sigma) was applied for a total of 30 min, including one fraction before, the fraction during and the fraction following the application of capsaicin plus drug. Superfusion was completed with a 30-min washout period using Krebs buffer alone. Immunoreactive CGRP release was measured over the 120-min period using radioimmunoassay quantification.

Radioimmunoassay

Following superfusion, samples as well as tubes containing known CGRP concentrations (1–300 fmol) were incubated with a C-terminally directed anti-CGRP antiserum (Dr Michael Iadarola, NIDCR, NIH, Bethesda, MD, USA) generated in rabbit. Following a 48-h incubation period at 4 °C, 100 µL of [¹²⁵I]CGRP_{28–37} (approximately 22 000 cpm) and 50 µL of goat antirabbit antibody coupled to magnetic beads (PerSeptive Biosystems, Framingham, MA, USA) were added. After a second 48 h incubation at 4 °C, bound CGRP was separated from free CGRP through immunomagnetic separation. Radioactive counts

were measured by a gamma counter. Samples containing known CGRP concentrations were used to generate standard curves. Unknown concentrations of iCGRP were determined using a logit-log transformation. The minimum detection limit for this assay is approximately 2–3 fmol/tube with 50% displacement occurring at 10–30 fmol/tube. All drugs used in these experiments were tested for effects on the standard curves and none were observed.

Statistics

Results for concentration response curves were analysed using nonlinear regression analysis and antagonist experiments were analysed using an unpaired, one-tailed Student's *t*-test (GraphPad Prism, San Diego, CA, USA). Statistical significance was accepted when the probability of a type I error was less than 5% ($P < 0.05$). Data on graphs represent mean $\pm$ SEM.

cDNAs and probe synthesis

Plasmid constructs containing the cDNA clones encoding the $\alpha 3$ (PCA48E), $\alpha 4$ (pA4.1) and $\alpha 6$ (pSN119) neuronal nicotinic receptor subunits were kindly provided by Dr Jim Boulter (UCLA, Los Angeles, CA, USA). For antisense probe synthesis, 1 μ g of plasmid DNA was linearized with the appropriate restriction endonuclease (Bam HI for $\alpha 3$, BstEII for $\alpha 4$ or NaeI for $\alpha 6$) and riboprobes were synthesized using an *in vitro* transcription kit (Promega, Madison, WI, USA) in the presence of 250 μ Ci each of [35 S]CTP α S and [35 S]UTP α S (Amersham, Arlington Heights, IL, USA) at 37 °C for 2h. Antisense probes corresponded to the last 504 bases C-terminal for $\alpha 3$, the last 864 bases C-terminal for $\alpha 4$ and the last 449 bases C-terminal for $\alpha 6$. Sense probes were also generated as controls. Probes were purified using G-50 spin column chromatography (Ambion, Austin, TX, USA), diluted to a concentration of 1×10^8 cpm/mL in hybridization buffer containing 50% formamide, 0.3 M NaCl, 10 mM Tris, 1 mM EDTA, $1 \times$ Denhardt's solution, 10% dextran sulphate, 50 μ g/mL yeast tRNA (Roche, Indianapolis, IN, USA) and 10 mM dithiothreitol and stored at –20 °C.

In situ hybridization immunohistochemistry

Rats were killed by decapitation; trigeminal ganglia were freshly dissected, frozen on dry ice, immobilized in mounting medium, cut into 20- μ m sections, mounted onto glass slides and stored at –80 °C. The sections were fixed for 1 h in ice-cold 10% formalin (3.7% formaldehyde w/v), permeabilized with 0.5% Triton X-100, acylated with 0.0025% acetic anhydride in 0.1 M triethanolamine, dehydrated and delipidated in alcohol and chloroform. Sections were then hybridized with *in vitro*-transcribed [35 S]-labelled riboprobes (75 μ L 1×10^7 cpm/mL per slide) complementary to mRNA encoding the $\alpha 3$, $\alpha 4$ or $\alpha 6$ neuronal nicotinic receptor subunits. Slides were coverslipped with Parafilm and incubated in a humidified chamber at 55 °C overnight. After RNase treatment and standard washing procedures, the tissue was blocked in phosphate-buffered saline containing 10% normal goat serum (Gibco, Rockville, MD, USA) and then incubated in rabbit-derived anti-CGRP antiserum (1:750; Peninsula, Belmont, CA, USA) or guinea pig-derived anti-VR1 antiserum (1:3000, Neuromics, Minneapolis, MN, USA) at 4 °C overnight. For single immunolabelling, tissue was then incubated for 1 h with Alexa Fluor 488-conjugated goat antirabbit or Alexa Fluor 488-conjugated goat antiguinea pig secondary antibody (1:300; Molecular Probes, Eugene, OR, USA), respectively. For double immunolabelling, tissue was first incubated with anti-CGRP antiserum overnight at 4 °C, followed by secondary antibody detailed above, then incubated with anti-VR1 antiserum overnight at 4 °C, followed by Alexa Fluor 594-conjugated goat antiguinea pig secondary antibody (1:300; Molecular Probes). Slides were then subjected to emulsion autoradiography using NTB-3 (Kodak, Rochester, NY, USA), developed and coverslipped.

Imaging

Images were acquired using an E600 microscope (Nikon, Melville, NY, USA) equipped with a Photometries Sensys black and white digital CCD camera and analysed using Metamorph V4.1 (Universal Image Corporation, Downingtown, PA, USA). Approximately 14–16 images at 20 $\times$ magnification were necessary to cover an entire trigeminal ganglion section. Cell size–frequency histograms were generated from 20 $\times$ images by measuring total area (which was later converted to diameter). Only cells that showed visible nuclei were included in the analysis. To determine total cell number or cells containing visible nuclei, the contrast of captured images was increased so that all cells, labelled and unlabelled by whichever method, could be seen. Cell counts for *in situ*-positive cells were performed by counting neurons that demonstrated above background silver grain labelling. Similarly, for immunopositive cells, counts were made on cells that were above background labelling using Metamorph's built-in scaling feature by establishing an average pixel value for background labelling and designating neurons above that pixel value as positive. Images were overlaid (dark field *in situ* hybridization and fluorescent immunohistochemistry image) to assess the presence of both nAChR mRNA and CGRP/VR1 immunoreactivity. In the case of triple labelling, three images were overlaid (dark field *in situ* and two fluorescent immunohistochemistry images). Dark field *in situ* and immunofluorescent images presented were pseudo-coloured using Metamorph. Percentages for each colocalization condition represent counts made on one section (14–16 images) from three separate animals while percentages for total nAChR mRNA-positive cells represent counts made on two sections each from three separate animals, wherein counted sections were > 100 μ m apart.

Results

Modulation of capsaicin-evoked immunoreactive calcitonin gene-related peptide release by nicotinic acetylcholine receptor agonists

Figure 1A shows the concentration-dependent potentiation of capsaicin-evoked iCGRP release by the prototypic nicotinic receptor agonist, nicotine. Peak effects ($E_{\max}$ 223%) of nicotine were seen at 10 mM and the concentration that produced a half-maximal effect (EC_{50}) was 556.5 μ M. To determine whether this effect was mediated by nAChRs, tissue was treated with the nAChR antagonist mecamylamine (10 μ M) and cotreated with 10 mM nicotine. As seen in Fig. 1B, the effects of nicotine were completely blocked in the presence of mecamylamine but not by the $\alpha 7$ nAChR antagonist methyllycaconitine (100 nM; not shown). Epibatidine, a high potency neuronal nicotinic receptor agonist, was also tested. The concentration–response curve for epibatidine is shown in Fig. 2A. Epibatidine evoked a concentration-dependent potentiation of capsaicin-evoked iCGRP release with maximal effects of 170.3% occurring at 10 nM (EC_{50} 317.2 pM). Co-administration of mecamylamine (10 μ M) in perfusion buffer again resulted in complete attenuation of the ability of epibatidine to potentiate capsaicin-evoked iCGRP release (Fig. 2B).

The effects of cytosine, a pharmacologically distinct nAChR agonist, on capsaicin-evoked iCGRP release are shown in Fig. 3A. Net peak iCGRP release was potentiated in a concentration-dependent manner with a maximal effect of 176.2% occurring at 1 μ M (EC_{50} 4.8 nM). Figure 3B shows complete attenuation of the potentiation of the capsaicin-evoked iCGRP release seen with cytosine when mecamylamine is included. Nicotine, epibatidine or cytosine, in the absence of capsaicin, did not significantly alter CGRP release nor did mecamylamine have any effect on basal or capsaicin-evoked release (data not shown).

Cell size distribution of nicotinic acetylcholine receptor subunit mRNA

To determine the profile of trigeminal ganglion neurons expressing nAChR mRNA, cell size–frequency histograms were generated for all three subunit mRNAs (Fig. 4A–C). Only cells whose nucleus was clearly visible were subjected to cell size measurements. For each subunit, random images were selected from those taken of trigeminal ganglion sections from three separate animals. The mRNA for the $\alpha 3$ nAChR subunit (Fig. 4A) was found mainly in neurons with an average diameter in the 20–30 μm (62 of 143 cells) and 30–40 μm (69 of 143 cells) range. Few cells with diameters in either the 10–20 μm (two of 143) or 40–50 μm (10 of 143) range were found and no $\alpha 3$ -positive cells were found in cell bodies with diameters larger than 50 μm . Very similar results were obtained for $\alpha 4$ (Fig. 4B) with the 20–30 μm (60 of 141) and 30–40 μm (69 of 141) diameter ranges containing the most mRNA-positive cells, while the 10–20 μm (two of 141) and 40–50 μm (10 of 141) ranges contained fewer positive cells. Again, no cells larger than 50 μm in diameter contained $\alpha 4$ mRNA. The distribution pattern for $\alpha 6$ mRNA (Fig. 4C) differed only slightly from that of $\alpha 3$ and $\alpha 4$. The 10–20 μm (two of 148) diameter range contained the least positive cells, the 20–30 μm (54 of 148) and 30–40 μm (70 of 148) ranges contained the most, while the 40–50 μm (22 of 148) range contained an intermediate number of positive cells, being slightly more than that of $\alpha 3$ or $\alpha 4$. As before, positive cells were not seen in neurons with a cell body diameter larger than 50 μm . No above background labelling was seen with sense control probes for $\alpha 3$, $\alpha 4$ or $\alpha 6$ (data not shown).

Colocalization of mRNA encoding nicotinic acetylcholine receptor with either calcitonin gene-related peptide or VR1 immunoreactivity

Results of combined *in situ* hybridization and immunohistochemistry on sections of rat trigeminal ganglion are detailed in Figs 5–7. Figure 5 shows the cellular distribution pattern of $\alpha 3$ mRNA with respect to CGRP or VR1 immunoreactivity. mRNA encoding the $\alpha 3$, $\alpha 4$ and $\alpha 6$ nAChR subunits was found, respectively, in 14, 9 and 19% of neurons, while immunoreactivity for CGRP or VR1 was found in approximately 36% of neurons (Table 1). Of those neurons expressing CGRP or VR1 immunoreactivity 5–9% coexpressed mRNA encoding either the $\alpha 3$ or $\alpha 4$ subunits, while almost twice as many (12–19%) coexpressed $\alpha 6$ mRNA (Table 2). Conversely, in neurons expressing $\alpha 3$ (Fig. 5) or $\alpha 4$ (Fig. 6) mRNA, 20–26% of these coexpressed CGRP or VR1 immunoreactivity and, in those expressing $\alpha 6$ mRNA (Fig. 7), the number coexpressing VR1 immunoreactivity reached 34% (Table 3). No difference was observed in the percentages for nAChR mRNA-positive cells in sections labelled using only *in situ* hybridization versus sections colabelled using immunohistochemistry (data not shown).

Co-localization of nicotinic acetylcholine receptor mRNA with both calcitonin gene-related peptide and VR1

To determine more definitively whether nAChR mRNA and CGRP and VR1 immunoreactivities are simultaneously coexpressed in the same neuron, triple-labelling experiments were performed (Figs 8–10). Figure 8 shows triple labelling for $\alpha 3$, Fig. 9 for $\alpha 4$ and Fig. 10 for $\alpha 6$, indicating that, for all three mRNA species, cells can be found that contain all three labels (a subunit mRNA, CGRP and VR1). Consistent with the higher colocalization percentages seen for $\alpha 6$ in Table 2, more triple-labelled cells can be seen in Fig. 10 than in Figs 8 and 9 although, for illustrative purposes, only one triple-labelled cell is highlighted in each figure.

Discussion

Capsaicin has been used extensively as an investigational tool for its ability to activate a subpopulation of unmyelinated and thinly myelinated, nociceptive sensory neurons (for

reviews, see Chapman *et al.*, 1961; Jansc *et al.*, 1967; Bevan & Szolcsanyi, 1990). More recently, the activation by capsaicin of a ligand-gated ion channel (VR1; also called TRPV1) expressed predominantly on C-fibre nociceptors has been described (Caterina *et al.*, 1997). Activation of these VR1 channels by capsaicin evokes the release of certain vasoactive neuropeptides, including CGRP and substance P (for review, see Holzer, 1991), which produce vasodilation and increased permeability of the endothelial membrane, thereby leading to erythema and oedema. The recent development of a model system in which to study capsaicin-stimulated CGRP release from rat buccal mucosa (Flores *et al.*, 2001) has enabled a more comprehensive evaluation of the mechanisms governing neuropeptide release from oral tissue in response to various stimuli. Consequently, this approach should allow for the determination of the potential roles played by these stimuli in the development of inflammatory disease within the oral cavity.

According to the World Health Organization, approximately 1.1 billion people smoke worldwide (<http://www.who.int>) and many more use smokeless tobacco. Not surprisingly, inflammation of the buccal, gingival and periodontal tissues is a major oral health problem among users of nicotine-containing tobacco products (see Haber *et al.*, 1993). Indeed, nicotine concentrations in the saliva of smokeless tobacco users has been measured to be as high as 10 mM (Hoffman & Adams, 1981), providing for the probable activation of multiple nicotinic receptor subtypes contained in oral tissues. The present studies were aimed at determining the effects of neuronal nicotinic receptor agonists on capsaicin-evoked CGRP release from rat buccal mucosa. In addition, experiments were performed to elucidate the presence of neuronal nicotinic receptor subunit transcripts in CGRP- or VR1-containing neurons whose cell bodies in the trigeminal ganglion innervate the oral cavity. These latter studies sought to suggest a possible mechanism for the increases in inflammation in oral tissues of tobacco users. As VR1 is a receptor for capsaicin (Caterina *et al.*, 1997), and stimulation of VR1-expressing neurons by capsaicin results in CGRP release (Holzer, 1991), the expression of functional nicotinic receptors on VR1/CGRP-immunoreactive neurons would provide a molecular basis upon which to hypothesize a direct action of nicotinic agonists on capsaicin-sensitive neurons in the modulation of CGRP release. By extension, this line of evidence supports a similar potential mechanism of action to explain the pro-inflammatory effects of nicotine or nicotine-containing tobacco products on the oral mucosa.

Here we provide the initial report on the distribution of $\alpha 6$ mRNA in sensory ganglia at the single cell level as well as the colocalization of the $\alpha 3$, $\alpha 4$ or $\alpha 6$ transcripts in CGRP- and/or VR1-immunoreactive neurons. As the origin of cell bodies whose axons innervate the buccal mucosa, the trigeminal ganglion represents a relevant anatomical location in which to visualize the cellular distribution of nAChR mRNA. Neuronal nicotinic receptor subtypes previously demonstrated in neurons of the trigeminal ganglion include the heteromeric $\alpha 3\beta 4$ and $\alpha 4\beta 2$ subtypes (Flores *et al.*, 1996) as well as α -bungarotoxin-binding sites, probably $\alpha 7$ receptors (Schechter *et al.*, 1978). Functional $\alpha 7$ -, $\alpha 3\beta 4$ - and $\alpha 4\beta 2$ -like receptors have also been measured in rat trigeminal (Liu & Simon, 1993; Liu *et al.*, 1998) and dorsal root (Genzen *et al.*, 2001) ganglia using electrophysiological techniques. In addition, reverse transcription–polymerase chain reaction studies have indicated that the trigeminal ganglion expresses mRNA encoding the $\alpha 6$ and, possibly, $\alpha 9$ subunits (Liu *et al.*, 1998; Keiger & Walker, 2000).

The diameter of most cells expressing nAChR subunit mRNA here was between 20 and 40 μm , implying that this expression occurs in unmyelinated or lightly myelinated C-fibre and A δ -fibre sensory neurons (Harper & Lawson, 1985), many of which are known to contain CGRP and express VR1 (Lawson *et al.*, 1993; Helliwell *et al.*, 1998; Tominaga *et al.*, 1998). To directly evaluate this possibility, double-labelling experiments were performed. Of cells that were positive for CGRP immunoreactivity, the percentage that colocalized $\alpha 3$, $\alpha 4$ or $\alpha 6$

nAChR subunit mRNA was approximately 7–12%. Colocalization of VR1-positive neurons with nAChR mRNA was similar but reached over 18% for $\alpha 6$. These observations, however, must take into account the current lack of information regarding the rate and extent to which CGRP or VR1 protein is trafficked out of the cell body. If transport occurs relatively rapidly and extensively after synthesis, then our colocalization studies might have underestimated the coexpression of these nAChR transcripts with CGRP and/or VR1. Additionally, depending on the sensitivity of the antibodies used to detect CGRP or VR1 immunoreactivity, the level of coexpression might have been similarly underestimated. In any event, the presence of these subunit mRNAs in VR1- or CGRP-expressing neurons constitutes supportive anatomical evidence concerning the ability of the nicotinic agonists tested to directly modulate CGRP release evoked by capsaicin. Insofar as the total amount of CGRP released by capsaicin in the presence of any of the nicotinic agonists tested was submaximal and a small fraction of the total pool (see Flores *et al.*, 2001), the relatively modest degree of colocalization between any given nAChR subunit, VR1 and CGRP is apparently sufficient and appropriate (i.e. a higher proportion of colocalizing neurons would have resulted in greater levels of enhanced release).

Although $\alpha 3\beta 4$ and $\alpha 4\beta 2$ receptors have been demonstrated previously in the trigeminal ganglion, it is not yet known whether the mRNA encoding the $\alpha 6$ subunit gives rise to functional nAChRs in this tissue; however, in combination with $\alpha 3$, $\beta 2$, $\beta 3$ or $\beta 4$ subunit, $\alpha 6$ forms functional receptor channels in chick retina or *Xenopus* oocytes (Gerzanich *et al.*, 1997; Kuryatov *et al.*, 2000; Vailati *et al.*, 1999). In addition, an $\alpha 6\beta 2$ site was recently proposed in dopaminergic neurons in the striatum, based on a loss of α -conotoxin MII binding in $\alpha 6$ knockout mice (Champtiaux *et al.*, 2002). Whether or not nAChRs that contain the $\alpha 6$ subunit are involved in the neurosecretory processes described here, nonetheless their present demonstration serves to increase the reported nAChR heterogeneity and distribution in sensory neurons. Indeed, the cellular localization of the $\alpha 6$ subunit suggests that the nAChRs that it contributes in forming may play a role in the modulation of inflammatory processes and/or nociception.

The studies detailed here in trigeminal ganglion neurons examined only the mRNA localization of certain nAChR α subunits. However, we may infer their translation and assembly in this structure based on previously cited studies documenting high affinity [3 H]-epibatidine-binding sites and nAChR-mediated electrophysiological responses. Taken together with the present data, demonstrating functional nAChRs in a tissue innervated by neurons originating in the trigeminal ganglion, we hypothesize that receptors formed by the subunits we have measured at the mRNA level are probably trafficked peripherally to the mucosa in the same way that α -bungarotoxin-binding nAChRs are transported peripherally from the dorsal root ganglion within the sciatic nerve (Ninkovic & Hunt, 1983).

However, there are non-neuronal cell types in buccal mucosa that express several receptors that are relevant to the present work. For example, human keratinocytes have recently also been shown to express functional VR1 (Inoue *et al.*, 2002; Southall *et al.*, 2003) and are known to express several nicotinic receptor subunits including $\alpha 3$, $\alpha 5$, $\alpha 7$, $\beta 2$ and $\beta 4$ (Grando *et al.*, 1995, 1996). However, these epithelial-derived cells do not elaborate CGRP and we are unaware of any other resident cells that do; therefore, these are not the target/source of capsaicin-evoked CGRP release described here. Moreover, there is considerable evidence to indicate that the origin of CGRP in orofacial tissues, including the buccal mucosa, is neuronal as well as capsaicin-sensitive and this has been extensively reviewed in the original work describing the model used in the present studies (Flores *et al.*, 2001). Thus, the simplest interpretation of the preponderance of experimental data is that the neurosecretory effects of nicotine and its congeners shown here are mediated by a direct

action on capsaicin-sensitive, CGRP-containing and nAChR-expressing trigeminal nerve terminals in the mucosa.

None of the nicotinic agonists used here possesses sufficient pharmacological selectivity to unequivocally implicate one or another nAChR subtype in mediating the potentiation of CGRP release. Nevertheless, the present data, taken together with previously published investigations, allow for reasonable speculation. That nicotine, epibatidine and cytosine each produced similar maximal effects (i.e. 70–123% potentiation) is consistent with the hypothesis that these drugs do so by activating the same receptor subtype. The fact that cytosine (Leutje & Patrick, 1991) and epibatidine (Buisson *et al.*, 2000) have been shown to be weak to moderate partial agonists at the $\alpha 4\beta 2$ subtype but are thought to be full agonists at the $\alpha 3\beta 4$ subtype would tend to implicate the latter, while the involvement of $\alpha 7$ -containing receptors can be largely ruled out based on the ability of mecamylamine and, at least in the case of nicotine, not methyllycaconitine to antagonize the actions of all of the agonists used. Although the EC_{50} of cytosine demonstrated here is inexplicably low (≈ 5 nM), perhaps owing to differential post-translational modifications and/or activation–inactivation kinetics of peripheral sensory neuronal nAChRs, that of nicotine (≈ 500 μ M) is virtually identical to published values for activating the $\alpha 3\beta 4$ subtype and is one to three orders of magnitude higher than that required to activate $\alpha 4\beta 2$ - or $\alpha 6$ -containing subtypes (Gerzanich *et al.*, 1995, 1997). Consistent with this view, no potentiation of capsaicin-evoked CGRP release was exhibited by the relatively selective $\alpha 4\beta 2$ agonist ABT-594 (Dussor and Flores, unpublished observations). Moreover, approximately 75% of all high affinity [3 H]-epibatidine binding in the trigeminal ganglion can be accounted for by receptors nominally comprised of $\alpha 3$ and $\beta 4$ subunits (Flores *et al.*, 1996). Finally, colocalization of CGRP and/or VR1 immunoreactivity with mRNA encoding the $\alpha 3$ or $\alpha 6$ subunit was relatively more frequent compared with the $\alpha 4$ subunit. However, these studies cannot rule out the possibility of activation of other subtypes not yet characterized in primary sensory neurons. The development and application of additional compounds with greater subtype selectivity or adaptation of these functional assays in tissues derived from nAChR subunit knockout mice would provide valuable insights toward the resolution of these questions.

With respect to the concentration of capsaicin used in the present studies (i.e. 100 μ M), this is an approximate EC_{80} (see Flores *et al.*, 2001) designed to provide a two-tailed window for observing either an inhibitory or an excitatory effect, whichever occurred. As detailed in the Discussion of the earlier work, we do not believe that this is the actual concentration reached at the neuronal terminal VR1 receptors in the mucosa, owing to the thickness and permeability of this tissue and the high lipophilicity of capsaicin. Moreover, the concentrations required to activate peripheral versus central VR1 are up to three orders of magnitude higher and the concentrations used here are consistent with those demonstrated to evoke CGRP release from paw skin (Kilo *et al.*, 2001). The fact that the effective concentrations of nicotinic agonists shown here are more in keeping with their published EC_{50} values probably reflects the fact that these compounds are more hydrophilic than capsaicin, thereby distributing more predictably in the extracellular fluid.

It is remarkable that, in contrast to other studies (Franco-Cereceda *et al.*, 1992; Hua *et al.*, 1994), none of the nAChR agonists used in this study were able to directly stimulate the release of iCGRP in the absence of capsaicin. However, these other studies utilized cultured neurons, visceral tissues (e.g. lung) or an alternate species (e.g. guinea-pig). This is not to say that there are no direct effects of nAChR activation on the trigeminal neuron terminals characterized here, as trigeminal or dorsal root ganglion neurons have been shown to be functionally activated by nAChR agonists. However, the only dependent measure assessed in the present studies was CGRP release. The primary neurotransmitters in the trigeminal ganglion have yet to be determined but immunohistochemical studies suggest glutamate as

at least one strong possibility (Inagaki *et al.*, 1987; Kai-Kai & Howe, 1991; Stoyanova *et al.*, 1998; Bae *et al.*, 2000). In fact, there are important differences with respect to the frequency/intensity of stimulation necessary to cause the release of glutamate, which is contained in small clear vesicles, and CGRP, which is predominantly found in large, dense core vesicles (for review see Langley & Grant, 1997). Accordingly, activation of nicotinic receptors may produce a frequency/intensity of stimulation necessary to release glutamate but not CGRP, although this remains to be determined. In this scenario, only in response to activation of the highly Ca^{2+} -permeable VR1 receptor by capsaicin and the additional influx of Ca^{2+} provided through nAChR activation would the release of CGRP be seen. Consistent with this rationale, nicotinic stimulation of motor neurons has no effect on acetylcholine release alone. It is only when motor neurons are stimulated electrically at sufficiently high frequencies that nicotinic agonists are able to modulate acetylcholine release (Bowman *et al.*, 1988; Vizi & Somogyi, 1989; Wessler, 1989).

Another consideration in terms of the net effect of vanilloid and nicotinic stimulation on CGRP release relates to the activation state of the respective receptors. In fact, it is likely that VR1 and/or the nAChRs being activated in the present studies become desensitized in the course of the 10-min period during which their agonists are being coapplied, although the resolution of this method does not provide a ready means for determining the relative contributions of these differentially activatable receptor species. That said, the preponderance of experimental evidence in this and other systems supports the conclusion that capsaicin-induced CGRP release arises from VR1 agonism. Similarly, and insofar as the effects of nicotinic agonists described here are mecamylamine-reversible, it is reasonable to conclude that they arise from nAChR agonism. Lastly, it would be interesting to determine the extent to which intracellular messengers engaged and mobilized by VR1 and/or nAChR activation may play a role. In any case, the ability of nAChR agonists, especially nicotine, to potentiate stimulated CGRP release, while having no effect on their own, suggests that they may predispose oral tissues to an enhanced neurogenic inflammatory response to other insults, such as thermal, chemical and mechanical tissue damage or infection.

In summary, we report here the potentiation of capsaicin-evoked CGRP release in buccal mucosa by the nAChR agonists nicotine, epibatidine and cytisine and provide evidence supporting the existence of several nicotinic receptor subtypes on CGRP-/VR1-containing neurons in trigeminal ganglia, the origin of sensory innervation to the buccal mucosa. Accordingly, these data provide a potential mechanism for the pro-inflammatory actions of nicotine on the oral tissues of tobacco users. Taken together, the present studies not only implicate sensory neuronal nAChRs in the mechanism by which the nicotine contained in tobacco products may contribute to inflammation within the oral cavity but also suggest that these receptors may constitute viable targets for the future development of a novel class of anti-inflammatory drugs.

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Abbreviations

CGRP	calcitonin gene-related peptide
iCGRP	immunoreactive CGRP
nAChR	nicotinic acetylcholine receptor

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Fig. 1.

(A) Potentiation of capsaicin-evoked immunoreactive calcitonin gene-related peptide (iCGRP) release by nicotine. Tissue was washed for 70 min with physiologic Krebs buffer (pH 7.4), followed by a 10-min superfusion of separate groups with capsaicin alone (100 μ M, $n = 6$) or capsaicin plus nicotine (100 μ M–0mM, $n = 5$ –6/group). Data are expressed as percentage control of net peak iCGRP release, calculated as the arithmetic difference between peak and basal release in fmol/sample. Concentration required to produce 50% effect (EC_{50}) was 556.5 μ M and maximal effect (E_{max}) was 123% over control. (B) Reversal of potentiation by nicotine of capsaicin-evoked iCGRP release by mecamlamine. Mecamlamine (10 μ M) was given 20 min before, 10 min during and 10 min following 10 mM nicotine + capsaicin superfusion. Groups were analysed by an unpaired, one-tailed Student's t -test ($*P < 0.01$) and data are represented as mean $\pm$ SEM.

Fig. 2.

(A) Potentiation of capsaicin-evoked immunoreactive calcitonin gene-related peptide (iCGRP) release by epibatidine. Tissue was washed for 70 min with physiologic Krebs buffer (pH 7.4), followed by a 10-min superfusion of separate groups with capsaicin alone (100 μ M, $n = 6$) or capsaicin plus epibatidine (100 pM–10nM, $n = 5$ –6/group). Data are expressed as percentage control of net peak iCGRP release, calculated as the arithmetic difference between peak and basal release in fmol/sample. Concentration required to produce 50% effect (EC_{50}) was 317.2 nM and maximal effect (E_{max}) was 70.3% over control. (B) Reversal of potentiation by epibatidine of capsaicin-evoked iCGRP release by mecamlamine. Mecamlamine (10 μ M) was given 20 min before, 10 min during and 10 min following 10 nM epibatidine + capsaicin superfusion. Groups were analysed by an unpaired, one-tailed Student's t -test (* $P < 0.05$) and data are represented as mean $\pm$ SEM.

Fig. 3.

(A) Potentiation of capsaicin-evoked immunoreactive calcitonin gene-related peptide (iCGRP) release by cytosine. Tissue was washed for 70 min with physiologic Krebs buffer (pH 7.4), followed by a 10-min superfusion of separate groups with capsaicin alone (100 μ M, $n = 6$) or capsaicin plus cytosine (1 nM–1 μ M, $n = 5$ –6/group). Data are expressed as percentage control of net peak iCGRP release, calculated as the arithmetic difference between peak and basal release in fmol/sample. Concentration required to produce 50% effect (EC_{50}) was 4.831 nM and maximal effect (E_{max}) was 76.2% over control. (B) Reversal of potentiation by cytosine of capsaicin-evoked iCGRP release by mecamlamine. Mecamlamine (10 μ M) was given 20 min before, 10 min during and 10 min following 1

μM cytosine + capsaicin superfusion. Groups were analysed by an unpaired, one-tailed Student's t -test ($*P < 0.05$) and data are represented as mean $\pm$ SEM.

Fig. 4. Cell size frequency histograms for (A) $\alpha 3$, (B) $\alpha 4$ or (C) $\alpha 6$ nicotinic acetylcholine receptor subunit mRNA. Only cells with a clearly identifiable nucleus were used for sizing. Cells were sized using Metamorph imaging software, counted and recorded in bins based on cell body diameter. Data are expressed as number of cells in each diameter range of the total cells counted for each subunit (total cells counted, 143 for $\alpha 3$, 141 for $\alpha 4$ and 148 for $\alpha 6$).

Fig. 5.

Colocalization of $\alpha 3$ nicotinic acetylcholine receptor mRNA with calcitonin gene-related peptide (CGRP) or VR1. A–C and D–F are from two separate sections. Large arrowheads, mRNA-positive cells; small arrowheads, immunopositive cells; arrows, double-labelled cells. Scale bars, 100 μm . A and D are dark-field *in situ* hybridization images for $\mu 3$ which have been pseudo-coloured red. Immunohistochemistry for CGRP or VR1 is shown in B and E, respectively. (C) Double labelling for $\alpha 3$ mRNA and CGRP; (F) double labelling for $\alpha 3$ mRNA and VR1.

Fig. 6. Colocalization of $\alpha 4$ nicotinic acetylcholine receptor mRNA with calcitonin gene-related peptide (CGRP) or VR1. A–C and D–F are from two separate sections. Large arrowheads, mRNA-positive cells; small arrowheads, immunopositive cells; arrows, double-labelled cells. Scale bars, 100 μ m. A and D are dark-field *in situ* hybridization images for $\alpha 4$ which have been pseudo-coloured red. Immunohistochemistry for CGRP or VR1 is shown in B and E, respectively. (C) Double labelling for $\alpha 4$ mRNA and CGRP; (F) double labelling for $\alpha 4$ mRNA and VR1.

Fig. 7.

Colocalization of $\alpha 6$ nicotinic acetylcholine receptor mRNA with calcitonin gene-related peptide (CGRP) or VR1. A–C and D–F are from two separate sections. Large arrowheads, mRNA-positive cells; small arrowheads, immunopositive cells; arrows, double-labelled cells. Scale bars, 100 μ m. A and D are dark-field *in situ* hybridization images for $\alpha 6$ which have been pseudo-coloured red. Immunohistochemistry for CGRP or VR1 is shown in B and E, respectively. (C) Double labelling for $\alpha 3$ mRNA and CGRP; (F) double labelling for $\alpha 6$ mRNA and VR1.

Fig. 8. Colocalization of $\alpha 3$ nicotinic acetylcholine receptor (nAChR) mRNA with calcitonin gene-related peptide (CGRP) and VR1. (A) Pseudo-coloured dark field *in situ* hybridization image for $\alpha 3$ nAChR mRNA; (B) immunolabelling for CGRP; (C) immunolabelling for VR1; (D) colabelling of all three. Arrows, cells positive for all three markers. Scale bars, 100 μ m.

Fig. 9. Colocalization of $\alpha 4$ nicotinic acetylcholine receptor (nAChR) mRNA with calcitonin gene-related peptide (CGRP) and VR1. (A) Pseudo-coloured dark field *in situ* hybridization image for $\alpha 4$ nAChR mRNA; (B) immunolabelling for CGRP; (C) immunolabelling for VR1; (D) colabelling of all three. Arrows, cells positive for all three markers. Scale bars, 100 μm .

Fig. 10. Colocalization of $\alpha 6$ nicotinic acetylcholine receptor (nAChR) mRNA with calcitonin gene-related peptide (CGRP) and VR1. (A) Pseudo-coloured dark field *in situ* hybridization image for $\alpha 6$ nAChR mRNA; (B) immunolabelling for CGRP; (C) immunolabelling for VR1; (D) colabelling of all three. Arrows, cells positive for all three markers. Scale bars, 100 μm .

Table 1

Expression of nicotinic acetylcholine receptor mRNA and calcitonin gene-related peptide (CGRP)/VR1 immunoreactivity in trigeminal neurons

Analysis	Labelled/total number [*]	(%) [‡]
α3 mRNA	1080/7421	(14.45 ± 1.54)
α4 mRNA	691/7445	(9.20) ± 1.48)
α6 mRNA	1436/7504	(19.21 ± 2.91)
CGRP IR	4011/11016	(36.37 ± 2.54)
VR1 IR	4145/11354	(36.54 ± 2.58)

^{*} Data for mRNA-positive cells represent counts of six entire sections (two from each of three animals). Data for immunoreactive (IR) cells represent counts of nine entire sections (three from each of three animals).

[‡] Data are expressed as mean ±SD.

Table 2

Co-expression of nicotinic acetylcholine receptor (nAChR) mRNA in CGRP-IR/VR1 IR neurons

nAChR mRNA	nAChR + CGRP-IR neurons/total CGRP-IR neurons [*]	(%) [‡]	nAChR + VR1 IR neurons/total VR1 IR neurons [†]	(%) [‡]
α3 nAChR	109/1276	(8.61 ± 0.66)	122/1428	(8.33 ± 1.99)
α4 nAChR	101/1363	(7.43 ± 1.76)	71/1257	(5.68 ± 1.40)
α6 nAChR	158/1372	(12.06 ± 2.95)	271/1460	(18.56 ± 6.69)

^{*} Data for CGRP-IR neurons coexpressing nAChR mRNA represent counts of three entire sections (one from each of three animals).

[†] Data for VR1-IR neurons coexpressing nAChR mRNA, represent counts of three entire sections (one from each of three animals).

[‡] Data are expressed as mean ± SD.

Table 3

Co-expression of CGRP-IR/VR1 IR in nicotinic acetylcholine receptor mRNA-positive neurons

Immunoreactivity	IR + $\alpha 3$ -positive/total $\alpha 3$ -positive *	(%) [§]	IR + $\alpha 4$ -positive/total $\alpha 4$ -positive [†]	(%) [§]	IR + $\alpha 6$ -positive/total $\alpha 6$ -positive [‡]	(%) [§]
CGRP	109/533	20.70 $\pm$ 2.45 [§]	101/376	26.78 $\pm$ 3.86	158/661	24.52 $\pm$ 3.99
VR1	122/547	22.10 $\pm$ 4.65	71/315	22.44 $\pm$ 2.64	271/775	34.14 $\pm$ 6.71

* Data for $\alpha 3$ mRNA-positive neurons coexpressing immunoreactivity for indicated analyte represent counts from three entire sections (one from each of three animals).[†] Data for $\alpha 4$ mRNA-positive neurons coexpressing immunoreactivity for indicated analyte, represent counts from three entire sections (one from each of three animals).[‡] Data for $\alpha 6$ mRNA-positive neurons coexpressing immunoreactivity for indicated analyte represent counts from three entire sections (one from each of three animals).[§] Data are expressed as mean $\pm$ SD.