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Responses of Cultured Rat Trigeminal Ganglion Neurons to Bitter Tastants

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Abstract

The initial steps in taste and olfaction result from the activation by chemical stimuli of taste receptor cells (TRCs) and olfactory receptor neurons (ORNs). In parallel with these two pathways is the chemosensitive trigeminal pathway whose neurons terminate in the oral and nasal cavities and which are activated by many of the same chemical stimuli that activate TRCs and ORNs. In a recent single unit study we investigated the responses of rat chorda tympani and glossopharnygeal neurons to a variety of bitter-tasting alkaloids, including nicotine, yohimbine, quinine, strychnine and caffeine, as well as capsaicin, the pungent ingredient in hot pepper. Here we apply many of these same compounds to cultured rat trigeminal ganglion (TG) neurons and measure changes in intracellular calcium $[Ca^{2+}]_i$ to determine whether TG neurons will respond to these same compounds. Of the 89 neurons tested, 34% responded to 1 mM nicotine, 7% to 1 mM caffeine, 5% to 1 mM denatonium benzoate, 22% to 1 mM quinine hydrochloride, 18% to 1 mM strychnine and 55% to 1 μM capsaicin. These data suggest that neurons from the TG respond to the same bitter-tasting chemical stimuli as do TRCs and are likely to contribute information sent to the higher CNS regarding the perception of bitter/irritating chemical stimuli.

Introduction

In a review of a meeting on the chemical senses entitled ' A Tale of Two Senses' (Ronnett and Payne, 1995), the two chemical senses that were featured were olfaction and gustation. Their selection was both expected, and indeed justified, since most research in the chemical senses has focused on these two senses. Although gustatory and olfactory neurons are activated (indirectly and/or directly) by chemical stimuli, it is seldom appreciated that neurons from the trigeminal nerve, which terminate in the oral and nasal cavities, also respond to a plethora of chemical stimuli (Silver, 1987; Cometto-Muniz and Cain, 1991). Such chemicals include capsaicin, nicotine, menthol, salts (e.g. NaCl), acids, aliphatic alcohols and a variety of other compounds that are generally classified as irritants (Cometto-Muniz and Cain, 1991; Wang *et al.*, 1993; Lundy and Contreras, 1994).

In contrast to the numerous advances made in elucidating the transduction mechanisms involved in olfaction and gustation (Zufall *et al.*, 1994; Lindemann, 1996), the mechanisms by which most chemical stimuli activate sensory trigeminal nerves are not well understood. Two reasons are that the terminals of these neurons are surrounded by epithelial cells and that they are very small. Taken together, both these factors make recording from the terminals of these neurons exceedingly difficult (Simon and Wang, 1993). Nevertheless, in many cases, the same chemicals that activate olfactory receptor neurons (ORNs) and taste receptor cells (TRCs) also activate trigeminal neurons, albeit, for the most part, at higher concentrations (Okuni, 1977, 1978; Cometto-Muniz and Cain, 1991; Lundy and Contreras, 1994; Walker *et al.*, 1996). In this paper we extend these studies to show that that neurons from the trigeminal ganglion (TG) are activated by a variety of bitter-tasting molecules, many of which were not heretofore thought to be trigeminal stimulants.

As an tastant, nicotine is interesting because, like most alkaloids, it has a bitter taste at low concentrations (Pfaffmann *et al.*, 1971). At higher concentrations, however, nicotine produces a burning sensation (Duner-Engstrom *et al.*, 1986; Jarvik and Assil, 1988). The burning sensation arises from the activation of nicotinic receptors on a subset of polymodal nociceptors in TG neurons that are also activated by capsaicin, the pungent ingredient in hot pepper (Fusco *et al.*, 1994; Liu and Simon, 1995). In this regard, trigeminal nerve activation by nicotine and capsaicin evokes a cascade of physiological responses that include: salivation, tearing, apnea, coughing and sneezing (Kuo *et al.*, 1992; Jinno *et al.*, 1994).

In a recent study we measured the single unit responses evoked by ten bitter-tasting stimuli from rat glosso-pharnygeal (GP) and chorda tympani (CT) neurons (Dahl *et al.*, 1997). The bitter compounds included five alkaloids

(quinine, strychnine, caffeine, nicotine, and yohimbine), two salts having a 'bitter' taste component ($MgCl_2$ and KCl), a 'bitter-tasting' amino acid (L-tyrosine), and two other bitter-tasting compounds [phenylthiocarbamide (PTC) and denatonium benzoate]. The results from these studies were summarized in a qualitative model for the coding of bitter tastants. Since many bitter compounds, especially the alkaloids, are lipophilic, and hence membrane-permeable, it is possible that on diffusing into the epithelium they will interact with the peripheral terminals of TG neurons that are embedded in the olfactory and lingual epithelia. Indeed, 3 mM quinine–HCl activates a subset trigeminal nerve fibers from the lingual branch (Pittman and Contreras, 1998). However, other than nicotine and quinine, it is not generally known whether TG neurons respond to other alkaloids or to the above mentioned compounds. To address the question we measured the relative changes in $[Ca^{2+}]_i$ in cultured TG neurons.

Materials and methods

All chemicals used were reagent grade (Sigma Chemical Co., St Louis, MO) dissolved in distilled water. The stimuli included 1 mM quinine–HCl, 1 mM caffeine, 0.1 mM nicotine,1 mM each L-tyrosine, yohimbine, PTC, strychnine–HCl and denato- nium benzoate, and 1 μM capsaicin. Stock solutions were made weekly and stored in the dark to slow chemical breakdown. Due to their low aqueous solubility, nicotine and capsaicin were dissolved in a small amount of dimethylsulfoxide (DMSO) prior to dilution in Krebs–Henseleit buffer (KH; see below). For comparison in single unit recordings from rat chorda tympani and glossopharnygeal neurons we use the same concentrations of yohimbine, strychnine and denatonium benzoate but higher concentrations of caffeine (50 mM), nicotine (1 mM) and quinine (10 mM) (Dahl *et al.*, 1997). The reasons for choosing lower concentrations of these last three stimuli in the main experiments was to avoid osmotic problems using hyper- osmotic solutions (caffeine and quinine), and to avoid producing a long-lasting increase in $[Ca^{2+}]_i$.

Trigeminal ganglia from 2-7p Sprague–Dawley rats were dissected aseptically and collected in Hank's balanced salt solution (HBSS) (Liu *et al.*, 1993, 1997). After washing twice in HBSS, the ganglia were diced into small pieces and incubated for 30–50 min at 37°C in 0.1% collagenase (Type XI-S, Sigma) in HBSS. Individual cells were dissociated by triturating the tissue through a fire-polished glass pipette, followed by a 10 min incubation at 37°C in 10 μg/ml DNase I (Type IV) in F-14 medium (Life Technologies, Gaithersberg, MD). The tissue was then centrifuged for 5 min at 70 *g* and the pellet was re-suspended in 2 ml of F-14 medium. Following a 10 min centrifugation at 800 *g* the cells were washed twice with F-14 medium the pellet was resuspended in DMEM supplemented with 10% fetal bovine serum and 200 ng/ml NGF. The cells were plated on poly-D-lysine- and laminin-coated glass coverslips (15 mm diameter) and cultured at 37°C in a water saturated atmosphere with 5% CO_2. At the beginning of each experiment, the neurons were placed in a chamber containing KH on an inverted microscope where the projected soma diameters were measured using a calibrated eyepiece. The composition (in mM) of KH was: NaCl 145, KCl 5, $CaCl_2$ 2.0, $MgCl_2$ 1.0, HEPES 10 and D-glucose 10 at pH 7.4. Experiments were performed at room temperature.

Intracellular calcium measurements

Measurements of intracellular Ca^{2+} were performed at room temperature in TG cells for 2–5 days. TG neurons adhered to glass coverslips were loaded at 37°C for 30 min with 10 μM calcium green-AM (Molecular Probes Inc.) in a bathing solution containing F-14 supplemented with 0.02% pluronic F-127 and 10% fetal calf serum. Prior to the experiment the cells were washed three times with KH and maintained in the dark for 15 min. Changes in intracellular Ca^{2+} were measured using an inverted Zeiss epifluorescence microscope equipped with a 40× oil immersion objective and fluorescence IC-100 camera (Photon Technology International Inc. South Brunswick, NJ). Fluorescence signals were digitized and stored on disk using a DT2867 integrated image processor system (Data Translation, Inc.) controlled by Axon Imaging 2.0 workbench software (Axon Instruments, Forster City, CA). Calcium green was excited at 488 nm and the fluorescence was measured at emission wavelength 510 nm. Images were acquired every 1–2 s. Since calcium green is a non-ratiometric Ca^{2+} indicator, the changes in intracellular Ca^{2+} were depicted as fluorescence intensity ratio (F/F_0) where F_0, the resting fluorescence, was determined at the beginning of each experiment by averaging 10 images prior to the addition of agonist. To exclude possible influences associated with changes in cell volume and/or position of the cell, both of these parameters were monitored throughout the experiments. We defined a response to a particular compound when $F/F_0 \geq 1.2$. The agonists (in KH) as well as KH were applied using using a multibarreled electrode (Adams and List Associated, Westbury, NY) that was placed ~20–50 μm from the cell.

Results

A total of 89 individual TG neurons were studied (Table 1). Nicotine (0.1 mM) was applied to all neurons. Of this total, nicotine evoked increases in fluorescence in ~33% of the neurons. The two other bitter stimuli that produced changes in a large percentage of neurons were 1 mM quinine (22%) and 1 mM strychnine (17.8%). All the other bitter stimuli produced changes in ≤7% of these neurons (Table 1).

Figure 1 shows the relative changes in fluorescence (F/F_0) in a neuron that was sequentially exposed to 10 s applications of 1 mM denatonium, 1 mM caffeine, 1 mM quinine–HCl, 1 mM strychnine and 100 μM nicotine.

Table 1 Activation of TG cells by bitter-tasting compounds

	Nicotine	Caffeine	Denatonium	Quinine	Strychnine	L-Tyrosine	Yohimbine	Capsaicin
Concentration (mM)	0	0.1	1	1	1	1	1	1
No. of cells tested	89	43	42	54	45	19	24	86
No. of cells responding	30	3	2	12	8	1	1	47
Percentage responding	33.7	7.0	4.8	22.2	17.8	5.3	4.2	54.7

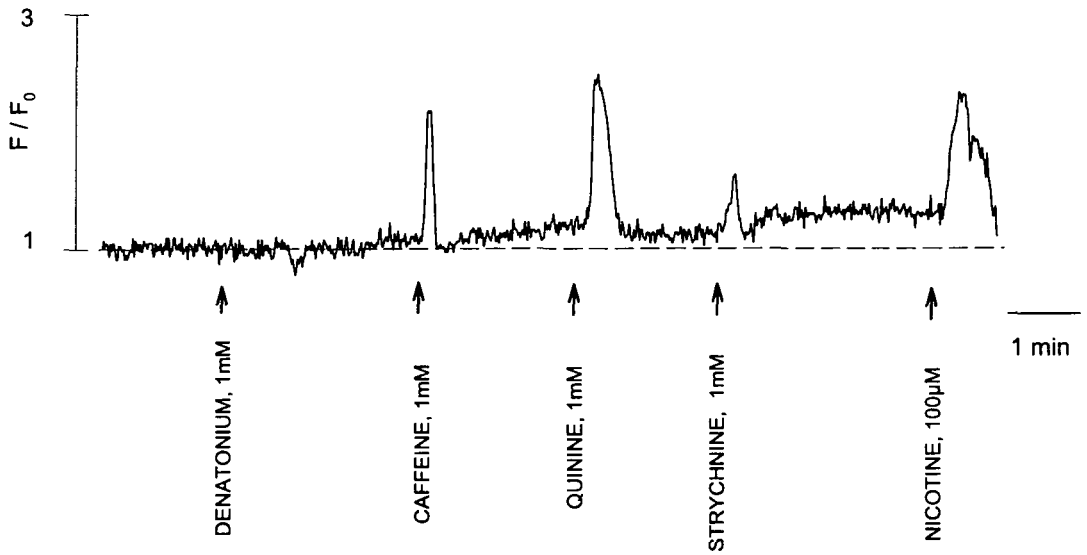

Figure 1 Changes in relative fluorescence (F/F_0) indicating the changes in $[Ca^{2+}]_i$ of single rat trigeminal neuron to several bitter stimuli (arrows). The stimuli were applied for 10 s and then the cell was washed with buffer. The calcium indicator was calcium green-AM.

Between applications the neurons were washed with KH buffer. It is evident that with the exception of denatonium all bitter tastants produced a transient increase in fluorescence. In fact, denatonium evoked a response in only 4.8% (2/42) of the cells tested (Table 1). These data show that not every bitter-tasting compound can activate all TG neurons, although in the neuron from which the data in Figure 1 came all the alkaloids tested evoked a response. We found that 75% of the neurons that responded to 0.1 mM nicotine also responded to 1 mM quinine (9/12), and 1 mM strychnine (6/8). In addition, all three neurons that were activated by 1 mM caffeine, were also activated by 1 mM nicotine (3/3).

Figure 2 shows the changes in fluorescence of a neuron that was sequentially exposed to 10 s applications of 1 µM capsaicin, 0.1 mM nicotine, 1 mM strychnine and 1 mM quinine. Between applications the cells were washed with KH buffer. Capsaicin activated a response that increased rapidly, but decreased ~60% upon washing. The calcium concentration remained elevated for several minutes during the wash. Of the subset of neurons that were activated by 1 µM capsaicin (see Table 2), 65% (15/23) were activated by 0.1 mM nicotine, 73% (8/11) were activated by 1 mM quinine, 67% were activated by 1 mM strychnine (4/6) and 100% (2/2) were activated by 1 mM caffeine and 1 mM yohimbine (1/1). What is evident is that capsaicin-sensitive neurons can be activated by a variety of stimuli not heretofore considered to be irritants.

Discussion

We found that a variety of bitter-tasting compounds activate neurons cultured from rat TG (Figures 1 and 2, Tables 1 and 2). For nicotine, this result was expected since extracellular recordings revealed that nicotine can activate TG neurons (MacIver and Tanelian, 1993; Wang *et al.*, 1993; Sekizawa and Tsubone, 1994; Walker *et al.*, 1996) and because rat TG neurons have many subtypes of nicotinic acetylcholine receptors (Wada *et al.*, 1989, 1990; Flores *et al.*, 1996; Walker *et al.*, 1996), including the α7 subtype (Liu *et al.*, 1996), which has a very high calcium permeability (McGhee and Role, 1995). For capsaicin, these results again were expected since receptors for capsaicin have been found on TG neurons (Szallasi and Blumberg, 1992) and extracellular recordings from rat TG revealed that they can be activated by capsaicin (Okuni, 1978; Chen *et al.*, 1997). Moreover, several studies using cultured TGs and DRGs

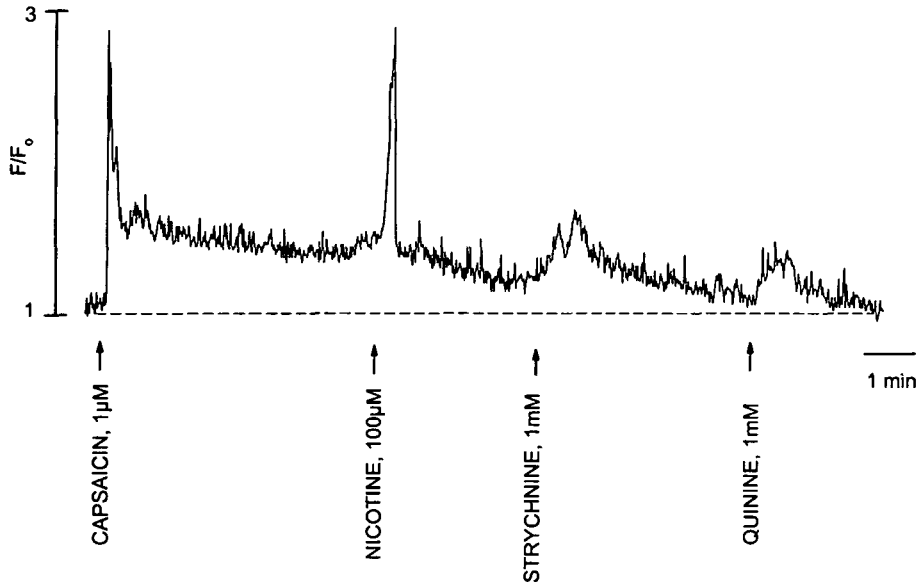

Figure 2 Changes in relative fluorescence (F/F_0) indicating the changes in $[Ca^{2+}]_i$ of single rat trigeminal neuron to capsaicin, nicotine, strychnine and quinine (arrows). The stimuli were applied for 10 s and then the cell was washed with buffer. The calcium indicator was calcium green-AM.

Table 2 Relationship between capsaicin and bitter-tasting compounds

	Nicotine	Caffeine	Denatonium	Quinine	Strychnine	L-Tyrosine	Yohimbine
Concentration (mM)	0.1	1	1	1	1	1	1
No. of cells tested	23	2	2	11	6	1	1
No. of cells that responded to capsaicin	15	2	1	8	4	0	1
Percentage that responded	65.2			72.7	66.7		

found that capsaicin increased $[Ca^{2+}]_i$ in about half of the neurons (Cholewinski *et al.*, 1993; Garcia-Hirschfeld *et al.*, 1995), a percentage consistent with the results obtained in this study (Table 1). What was not previously known was that a variety of other bitter-tasting chemical stimuli that have been shown to activate TRCs, such as strychnine, denatonium and caffeine, could activate TG neurons. These compounds, in fact, are unlikely to be specific for TRCs or even selective types of sensory neurons, since these same compounds have been shown to change the membrane potential of neuroblastoma (N-18) cells (Kurihara *et al.*, 1994).

An interesting issue arising from this study is that a variety of alkaloids, such as strychnine, quinine and caffeine, can increase $[Ca^{2+}]_i$ in capsaicin-sensitive neurons. Numerous studies have shown that chemical stimuli such as nicotine and capsaicin that increase $[Ca^{2+}]_i$ in sensory neurons can induce peptide release from nerve terminals even without the necessity of evoking action potentials (Lou *et al.*, 1992; Lundberg *et al.*, 1994). Since capsaicin-sensitive neurons are primarily polymodal nociceptors that can produce afferent (via propagated action potentials) and efferent (via peptide release) responses, it follows that a variety of bitter-tasting compounds, such as quinine and strychnine, may produce physiological effects similar to those evoked by nicotine (coughing, vasodilation, apnea) (Jinno *et al.*, 1994). Some of these responses, such as vasodilation, may in fact modulate taste responses (Hellekant, 1977). In this regard high concentrations of quinine which, in addition to being bitter, may also be irritating, a characteristic that may contribute to it evoking aversive behaviors (e.g. gaping).

Although the responses of rat TG neurons to most bitter compounds have not been reported, we note that whole-nerve recordings from rat lingual nerves failed to detect a response to quinine (Kawamura *et al.*, 1968; Sostman and Simon, 1991). However, in multiunit recordings the responses to quinine may be masked, most likely because of the large number of chemically insensitive Aβ-fibers (Wang *et al.*, 1993). In contrast, single unit responses of rat lingual nerves to ≤3 mM quinine were initially excitatory and then inhibitory, and they also modulated the response of some thermally sensitive neurons (Lundy and Contreras, 1994; Pittman and Contreras, 1998).

Also Bossut *et al.* (1997) found in single unit recordings from rat spinal trigeminal nucleus that 10 mM quinine applied to the tongue or cornea activated a subset of these neurons. These data, taken together, show that quinine can activate neurons in the rat trigeminal system.

The fact that neurons from the trigeminal nerve may be activated by quinine suggests that general sensory GP neurons may also respond to quinine. If this is the case, then one possible reason that quinine evokes a larger glossopharnygeal than chorda tympani response (Frank, 1991; Dahl *et al.*, 1997) may be that it also activates general sensory GP neurons. In this regard it is important to recall that, unlike chorda tympani neurons, which are all special sensory neurons, the lingual branch of the GP nerve contains both special and general sensory neurons. Since single unit GP recordings do not, in and of themselves, provide information regarding the destination of the neuron, many of the quinine-sensitive single unit GP responses may not be associated with TRCs. In any event, it is clear that activation of these general sensory neurons, whether trigeminal or general sensory GP, could give rise to irritating perceptions, especially if they also activate capsaicin-sensitive polymodal nociceptors.

We have not investigated the mechanisms by which these bitter-tasting compounds activate TG neurons to increase $[Ca^{2+}]_i$. This was not, in fact, our goal. Our goal was to determine whether a variety of stimuli, heretofore classified as bitter tastants, can activate TG neurons at the same or even lower concentrations as were used to evoke responses from rat chorda tympani and glossopharnygeal neurons (Dahl *et al.*, 1997). This we have achieved. The issue of whether most bitter tastants also have an irritating (trigeminal) component is ambiguous, especially at high concentrations. This is because most of the psychophysical literature on bitter taste usually reports on the intensity of the bitter taste sensation and questions regarding the irritating quality of bitter tastes are not generally reported. It is our hope that the data presented in this paper will stimulate psychophysical researchers to investigate the trigeminal contribution associated with bitter (irritating) taste sensations.

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