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Lack of Quinine-evoked Activity in Rat Trigeminal Subnucleus Caudalis

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Abstract

Conflicting reports exist regarding the ability of quinine to activate neurons in the trigeminal system. We used the complementary approaches of single-unit electrophysiology and c-fos immunohistochemistry to investigate whether quinine (100 mM) activates chemonociceptive cells in the brainstem trigeminal subnucleus caudalis (Vc). In electrophysiological experiments, 38 units responded to noxious mechanical, thermal and chemical (200 mM pentanoic acid) stimuli applied to the tongue with an increase in firing rate; none responded to lingual quinine whether the quinine was presented before or after application of pentanoic acid. In the c-fos immunohistochemical experiment, both quinine and water elicited equivalent levels of fos-like immunoreactivity (FLI) in dorsomedial Vc that were significantly lower than the level of FLI evoked by pentanoic acid. These data collectively indicate that quinine does not elicit activity in chemonociceptive Vc neurons.

Introduction

Sensations of taste and oral irritation are thought to be processed separately via gustatory and somatosensory pathways, respectively. Indeed, prototypical taste stimuli applied to the tongue activate peripheral neurons that project to the brainstem taste relay, the nucleus of the solitary tract (NTS) (Herness and Gilbertson, 1999; Smith and St John, 1999). In contrast, irritant stimuli excite small diameter nociceptive neurons that project to the caudal aspect of the brainstem trigeminal complex (Vc) (Sostman and Simon, 1991; Carstens *et al*., 1998). Interestingly, the tastants NaCl and citric acid, when delivered to the tongue at high concentrations, elicit neuronal activity in Vc (Carstens *et al*., 1998; Sudo *et al*., 2002), as well as sensations of irritation (Green and Gelhard, 1989; Gilmore and Green, 1993; Dessirier *et al*., 2000a, 2001).

Recently, several reports have indicated that quinine is able to activate lingual trigeminal afferents (Lundy and Contreras, 1994; Pittman and Contreras, 1998) or increase intracellular calcium in trigeminal ganglion neurons (Liu and Simon, 1998). Despite this evidence, there have been no reports of quinine inducing sensations of irritation when applied to the human tongue in psychophysical experiments. Indeed, in human studies directly addressing this issue, no subject reported quinine as having any sensation other than bitter (B.G. Green, personal communication). The physiological and psychophysical results, therefore, appear dichotomous.

It is commonly accepted that taste provides important information regarding nutritional and qualitative aspects of ingested items [for review, see Scott and Verhagen (Scott and Verhagen, 2000)]. Chemesthesis, on the other hand, conveys information regarding the presence of noxious and potentially dangerous chemicals (Green *et al*., 1990; Green and Lawless, 1991). Thus it makes adaptive sense that poisonous alkaloids, which typically taste bitter, might activate both taste and trigeminal pathways. In this way, parallel pathways would ensure the detection of deleterious chemicals, in turn initiating oromotor responses that result in their rejection. In the current study we employed two complementary techniques, electrophysiology and c-fos immunohistochemistry, to explore the possibility that quinine excites neurons in Vc.

Materials and methods

Animals

Thirty-eight male Sprague–Dawley rats (Simonsen, Gilroy, CA) weighing ~450 g were used in these experiments. They were housed two per cage in a vivarium maintained on a 12 h:12 h light:dark cycle (light on at 7 a.m.) at ~21°C and allowed food and water *ad libitum*. All procedures were in accordance with the NIH animal welfare guidelines and approved by the UC Davis Animal Use and Care Committee.

Experiment 1: electrophysiology

Surgery

Rats (n = 20) were anesthetized with thiopental (55 mg/kg; i.p.) and core body temperature was maintained at ~37°C by placing the animal under a heating lamp. The jugular vein was cannulated to allow constant infusion of thiopental (10 mg/kg per h) and a tracheal cannula was placed to assist breathing. A dorsal midline incision was made and the caudal brainstem and upper cervical spinal cord exposed by removal of the atlanto-occipital membrane and caudal portion of the occipital bone. Animals were fixed into a stereotaxic frame (David Kopf Instruments, Tujunga, CA) with their head ventroflexed (to expose obex) and the upper cervical spine immobilized with a vertebral clamp. The dura mater was removed and agar (Difco, Detriot, MI) poured over the brainstem. After hardening, an opening was cut through the agar in an area overlying Vc and filled with 0.9% saline. Finally, a small clip was placed over the incisors to hold open the mouth and allow access to the anterior tongue. Physiological saline was applied to the lingual surface to prevent desiccation.

Recording

A Teflon-insulated tungsten recording electrode (~10 MΩ; F. Haer Inc. Brunswick, ME) was advanced into the brainstem (~1.5 mm lateral to obex) using a hydraulic microdrive (David Kopf Instruments). Single unit recordings were made from nociceptive neurons in superficial laminae (<300 μm) of the dorsomedial Vc having receptive fields on the ipsilateral anterior tongue. Extracellular activity was amplified, digitized and fed to a computer for later analysis at which time unitary action potentials were discriminated and counted by custom software (Forster and Handwerker, 1990).

Stimulation

Vc units responsive to heat (55°C) and pinch were isolated and further tested for chemosensitivity using pentanoic acid (200 mM dissolved in deionized water; Sigma Chemicals, St Louis, MO; pH 3.0). Only chemonociceptive units were selected for further study. Pentanoic acid was applied via syringe as a bolus (0.3 ml) to the dorsal surface of the tongue and rinsed 90 s later with deionized water (~2 ml). Quinine–HCl (100 mM dissolved in deionized water, pH 5.8; Sigma Chemicals) was applied in an identical manner. This concentration, which was equal to (Kawamura *et al.*, 1968; Sostman and Simon, 1991) or higher (Lundy and Contreras, 1994; Liu and Simon, 1998; Pittman and Contreras, 1998) than concentrations used in other experiments, was selected to increase the chance of exciting units. All chemicals were applied at room temperature.

Chemical stimuli were applied successively to the lingual surface at interstimulus intervals of 5 min. Pentanoic acid was applied twice, followed by quinine, followed again by pentanoic acid. Thus, the duration of each experiment lasted <30 min. In 11 animals, recordings were made from additional units, usually on the same side, isolated and tested in the same manner (average 2–3 units/rat). A minimum of 30 min elapsed between successive recordings in animals from which multiple units were isolated.

Using the current stimulation parameters, pentanoic acid neither sensitizes nor desensitizes subsequent noxious-evoked responses of Vc neurons (Dessirier *et al.*, 2000b; Sudo *et al.*, 2002) and we believe that it was unlikely to affect Vc responsiveness to quinine. Nevertheless, to eliminate this possibility, we reversed the order of stimulus presentation so that quinine preceded pentanoic acid. This required us to test responses of Vc neurons to quinine prior to any other chemical stimulation of the tongue. To increase the likelihood that the Vc neuron was chemonociceptive, in each of seven rats, we selected a neuron that responded to the noxious thermal stimulus because there is a good correspondence between heat- and pentanoic acid-sensitivity (Dessirier *et al.*, 2000b). Heat-responsive Vc neurons were then tested with quinine delivered in the same manner as described above, followed 5 min later by pentanoic acid to confirm that the Vc unit was chemonociceptive.

Histology

Following the completion of the last recording for each animal, an electrolytic lesion was made at the recording site by passing current (6 V DC) through the microelectrode for 45 s. Animals were killed with an overdose of thiopental (~100 mg/kg; i.v.) and the brains were removed and fixed in 10% formalin. Not less than 1 week later, the brains were frozen, cut into 50 μM sections, collected onto glass slides and counterstained with neutral red. Lesions were identified under a light microscope and collectively plotted onto a representative brainstem section (Figures 2C and 3B).

Data analysis

To determine if pentanoic acid or quinine elicited activity above baseline levels, each unit's response was integrated into 1 or 60 s bins (baseline: 0–60 s, initial response: 61–120 s, late response: 121–180 s) and analyzed using analysis of variance (ANOVA; animal and time as main effects). To determine if the magnitude of Vc responses elicited by pentanoic acid was different from that elicited by quinine, unit responses to each of the stimulus trials were integrated (60–150 s) and subjected to ANOVA (animal and trial as main effects). A $P < 0.05$ was taken as significant and all data are presented as means ± SE.

Experiment 2: immunohistochemistry

Stimulation

Animals (n = 18) were anesthetized with pentobarbital (65 mg/kg; i.p.) and a clip was placed over the upper and lower incisors to keep the mouth open and provide access to the tongue. Animals received either quinine (100 mM; n = 6), pentanoic acid (200 mM; n = 6) or deionized water

(n = 6) delivered to the anterior third of the tongue by syringe. For all groups, the stimulus was applied gently as a bolus (~0.3 ml) at time 0 and again 15 min later. To minimize any confounding mechanosensory-induced labeling, stimuli were not rinsed. The clip was gently removed 20 min after the last stimulus and the animals were closely monitored until they were killed by perfusion.

Staining

Two hours after the onset of lingual stimulation, each animal was perfused through the heart with 250 ml of phosphate-buffered saline (PBS) followed immediately by 500 ml of 4% paraformaldehyde. The brains were removed, post-fixed in 4% paraformaldehyde for ~24 h and transferred to a 30% sucrose solution for cryoprotection. One to 2 days later, the brains were frozen, cut into 50 µm sections and processed for c-fos immunohistochemistry as previously described (Simons *et al.*, 1999). Briefly, the sections were first blocked with 3% normal goat serum (in PBS with 0.3% Triton X-100) and then exposed to the primary c-fos antibody (diluted 1:50 000; Arnel Products Inc., New York, NY) for 24–36 h. The primary antibody was removed and the sections washed followed by application of the secondary biotinylated goat anti-rabbit antibody (Vector Laboratories, Burlingame, CA). One hour later, this antibody was removed, the sections were washed again, subjected to the avidin–biotin–peroxidase reaction and cell nuclei expressing fos-like immunoreactivity (FLI) were stained black by a nickel diaminobenzidine reaction. Brainstem sections were mounted and coverslipped on glass slides for later microscopic analysis.

Data analysis

All sections were examined under the light microscope (Nikon E-400) and cell nuclei displaying black FLI were counted bilaterally between the level of the pyramidal decussation caudally up to the rostral pole of the nucleus of the solitary tract (NTS) in five regions of interest: (i) dorsomedial Vc, (ii) ventrolateral Vc, (iii) the caudal NTS up to the level of the area postema, (iv) the gustatory NTS from the area postrema (AP) through the rostral pole and (v) the ventrolateral medullary area dorsal to the lateral reticular nucleus. The dorsomedial Vc region was restricted to the area within the gray matter and 50 µm medial to it. The ventrolateral Vc region was restricted to the gray matter and fiber bundles within 50 µm of the medial gray matter border. These restrictions delimited the Vc regions from the more medially situated NTS dorsally and the ventrolateral medullary area ventrally (Carstens *et al.*, 1995). Counts of FLI were made in each of the five regions bilaterally at 150 µm intervals. The investigator who did counts of FLI was blinded as to the experimental treatment. For each treatment group, total FLI in each region of interest was divided by the number of sections examined to yield mean FLI/section. The numbers of FLI expressing neurons in each area of interest were compared between treatment groups using ANOVA (treatment group as main effect) followed by *post hoc* LSD tests; a P value of <0.05 was considered to be significant. For illustrations, selected sections were imaged with a color video camera (Dage MTI DC-330) using Scion Image software and superimposed on line drawings of sections taken from the atlas of Paxinos and Watson (Paxinos and Watson, 1998) to plot distributions of FLI (Figure 4).

Results

Electrophysiology

Thirty-eight units responded to noxious heat (54°C) and pentanoic acid. Of these, 26 responded to non-noxious mechanical stimulation and were categorized as wide dynamic range (WDR) type whereas 12 responded only to noxious mechanical stimulation (pinch) and were categorized as nociceptive-specific. Spontaneous activity in these units was generally low and seldom exceeded 5 Hz. Mechanical receptive fields were limited to the ipsilateral tongue and occasionally included the ipsilateral lip. An example of such a recording is shown in Figure 1.

The initial pentanoic acid stimulus elicited a mean response in these neurons that was significantly [$F(2,56) = 40.2$; $P < 0.001$] higher than baseline activity and peaked ~45 s following the stimulus onset (Figure 2A). Subsequent pentanoic acid trials elicited responses that were of equal magnitude [LSD: P(1 versus 2) = 0.248, P(1 versus 3) = 0.102, and P(2 versus 3) = 0.609] and temporal structure (Figure 2B,D). Lingual quinine application, however, did not evoke activity above basal levels (Figure 2C) when analyzed in 1 s [$F(209,5852) = 1.113$; $P = 0.130$] or 60 s bins [$F(2,56) = 0.1$; $P = 0.938$]. Indeed, the magnitude of the mean quinine response was not different from pre-stimulation baseline but was significantly smaller than any response elicited by pentanoic acid stimulation [$F(3,84) = 42.4$; $P < 0.001$].

In seven heat-responsive units, quinine was tested prior to pentanoic acid. In these units, lingual quinine application did not evoke activity above basal levels [$F(2,12) = 0.888$, $P = 0.437$; Figure 3A] whereas the pentanoic acid stimulus elicited activity in each of the seven units that was, on average, significantly [$F(2,12) = 12.2$, $P < 0.001$] higher than spontaneous levels (Figure 3B). In these seven units, pentanoic acid evoked significantly [$F(1,12) = 26.8$, $P < 0.001$] larger responses compared with quinine, confirming that quinine applied either before or after pentanoic acid does not excite chemonociceptive Vc units.

Immunohistochemistry

Figure 4 shows individual examples of the distribution of FLI (dots) at three representative brainstem levels following application of pentanoic acid (A), quinine (B) or water (C). There were no significant differences in counts of FLI

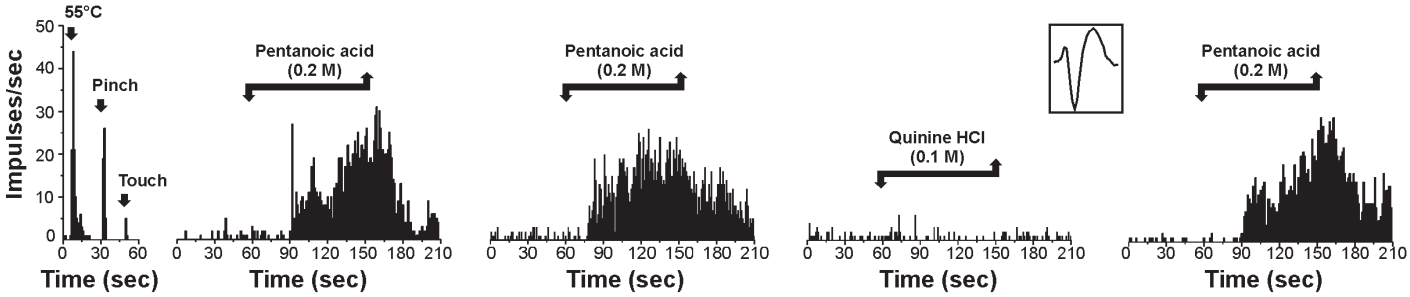

Figure 1 Individual example of a Vc neuronal response to pentanoic acid and quinine. Shown are peristimulus time histograms (PSTHs; bin width: 1 s) of a unit's response to noxious heat (55°C; first panel), pinch (first panel), touch (first panel), three applications of pentanoic acid (200 mM; second, third and fifth panel) and one application of quinine (100 mM; fourth panel) for 90 s. The interstimulus interval was 5 min. Downward arrow indicates stimulus onset and upward arrow indicates deionized water rinse. Inset shows the action potential waveform.

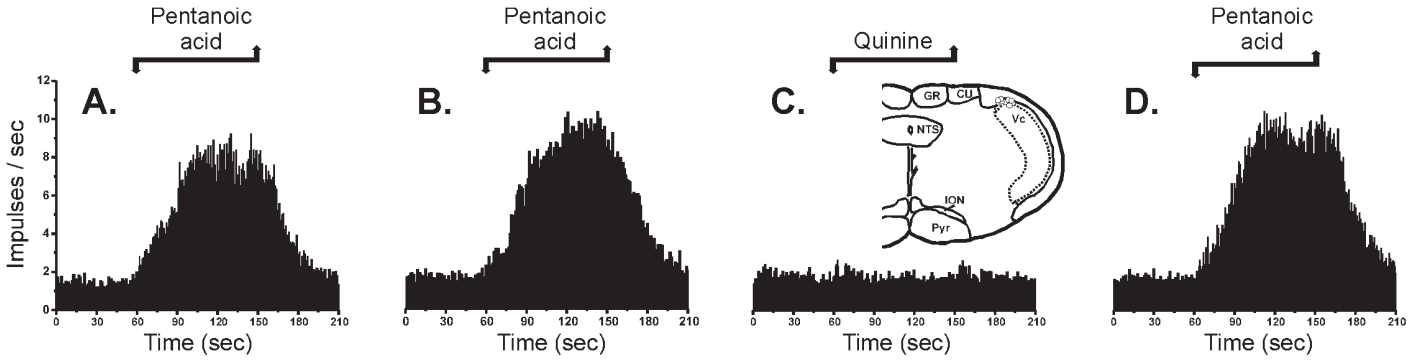

Figure 2 Averaged response of 34 units to pentanoic acid and quinine. **(A, B, D)** Averaged PSTHs (1 s bin width) of Vc neuronal response to pentanoic acid (200 mM) delivered for a duration of 90 s (indicated by bars with arrows) to the dorsal anterior lingual surface. Error bars omitted from PSTHs for clarity. Note lack of any sensitizing or desensitizing effect of repeated application. **(C)** Averaged response of these same units to quinine. Note the lack of any quinine-evoked activity. **(E)** Vc recording sites (circles) plotted on a representative brainstem section. Abbreviations are given in the legend to Figure 4.

between the quinine and water (vehicle control) treatment groups for any of the brainstem regions analyzed (Figure 5). In the dorsomedial aspect of Vc, corresponding to where our electrophysiological recordings were made, pentanoic acid elicited significantly [$F(2,15) = 14.6$; $P < 0.001$] higher mean counts of FLI compared with quinine or water (Figure 5, Vc dm). Using identical methods, we previously reported that there was no FLI in dorsomedial Vc in control animals not receiving any stimulus to the tongue (Simons *et al.*, 1999). Thus, the FLI that was seen following quinine and water (~9) might reflect a low level of activation of the Vc neurons by mechanical and/or thermal components of the stimulus. There were no significant differences among treatment groups for any of the other brainstem regions, including the rostral aspect of NTS (Figure 5; NTSr). In particular, we did not observe increased FLI at the rostral pole of NTS (Figure 4A–C, uppermost sections) following application of any of the stimuli.

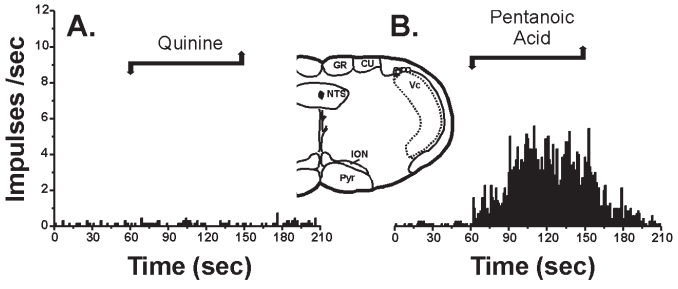

Figure 3 Averaged response of seven heat-sensitive units tested with quinine prior to pentanoic acid stimulation. **(A)** Averaged PSTH (1 s bin width) of Vc neuronal response to quinine (100 mM) delivered for 90 s to the dorsal lingual surface. Note lack of any quinine-evoked response. **(B)** Averaged response of these same units to pentanoic acid. Inset shows the recording sites (circles) plotted as in Figure 2C (abbreviations given in legend to Figure 4).

Discussion

Complementary immunohistochemical and electrophysiological methods were used to test the hypothesis that the bitter tastant quinine would activate chemonociceptive neurons in the brainstem trigeminal complex. Presently, we found that a high concentration of quinine applied to the tongue was unable to excite individual chemonociceptive Vc units or evoke FLI in dorsomedial Vc.

Early studies indicated that a high concentration of quinine was incapable of exciting lingual nerve fibers when

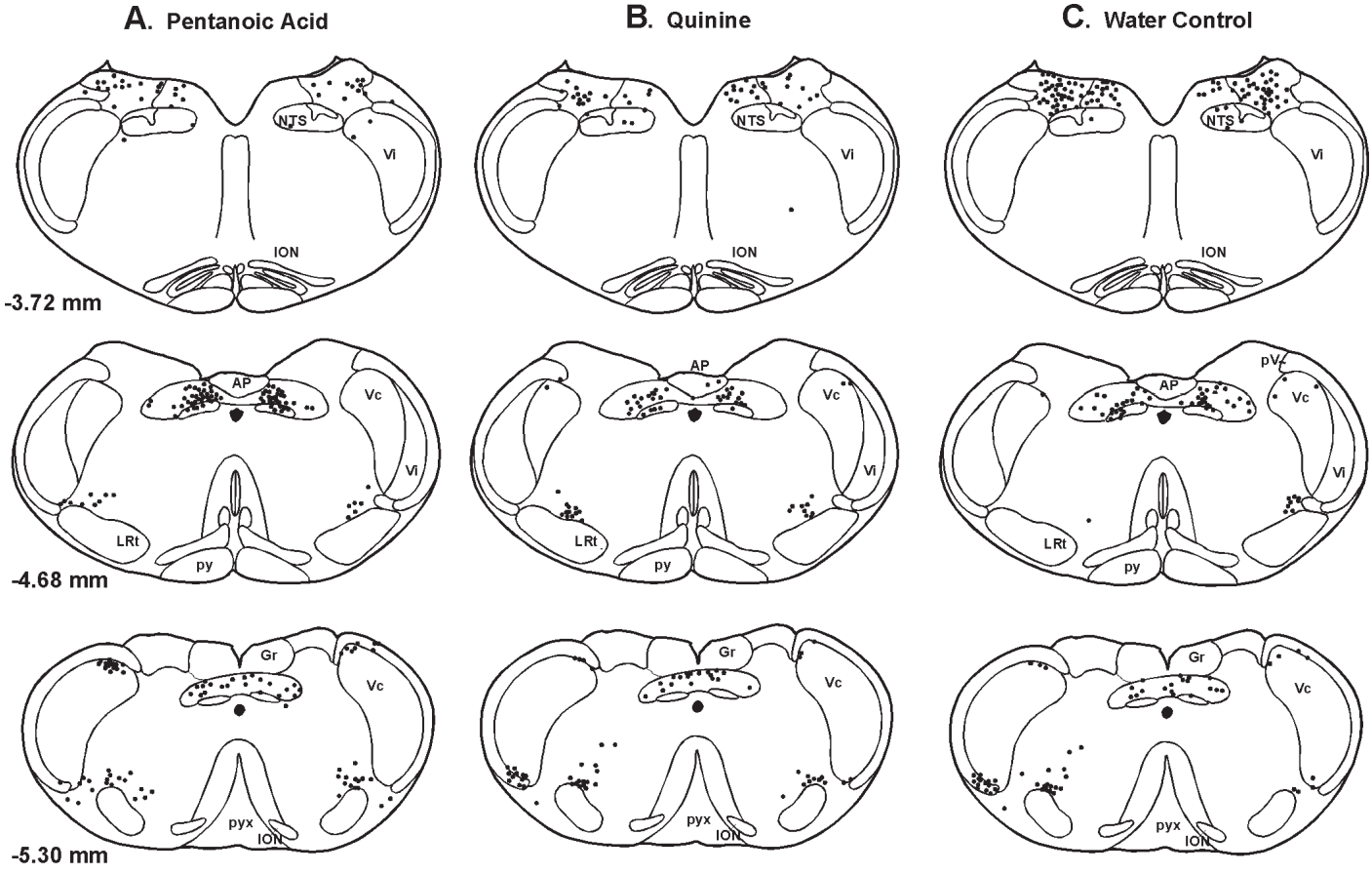

Figure 4 Brainstem distribution of FLI elicited by pentanoic acid, quinine or deionized water stimuli. **(A)** Representative transverse brainstem sections (taken from the atlas of Paxinos and Watson (Paxinos and Watson, 1998) showing distribution of FLI caudally at the level of the pyramidal decussation (lower figure) through the rostral pole of the NTS (upper figure) in an animal receiving pentanoic acid (●). Numbers to left indicate stereotaxic coordinates relative to the interaural line. **(B)** Representative sections taken from an animal receiving oral quinine. Format as in (A). **(C)** Brainstem sections from a representative animal previously stimulated with deionized water. Format as in (A). Abbreviations: AP, area postrema; Gr, gracile nucleus; ION, inferior olivary nucleus; LRt, lateral reticular nucleus; NTS, nucleus of the solitary tract; pV, paratrigeminal nuclei; py, pyramid; pyx, pyramidal decussation; Vc, trigeminal subnucleus caudalis; Vi, trigeminal subnucleus interpolaris.

applied to the tongue (Kawamura *et al*., 1968) even though these same fibers responded well to high salt concentrations. Subsequent studies confirmed that lingual fibers were responsive to high concentrations of salts and acids but failed to respond consistently to quinine (Sostman and Simon, 1991). However, these results are contradicted by more recent evidence indicating that quinine elicits relatively small (±2 spikes from baseline) and transient (within the first 2 s) responses in thermally sensitive trigeminal afferents (Pittman and Contreras, 1998). Similarly, quinine was shown to increase intracellular calcium in cultured trigeminal ganglion neurons (Liu and Simon, 1998). These studies support behavioral data indicating an increase in rat quinine rejection-thresholds following trigeminal deafferentation (Jacquin, 1983). However, in the behavioral study, the increase in quinine-rejection thresholds was thought to reflect a failure on the part of the rat to respond to subtle gustatory cues following massive orosensory alterations as well as changes in taste bud morphology (Jacquin, 1983) as opposed to a loss of trigeminally mediated quinine sensitivity.

Prior calcium imaging work on trigeminal ganglion neurons showed that ~73% of cells responding to capsaicin also responded to quinine (Liu and Simon, 1998) suggesting that these cell types are involved in chemonociception. In the present study, we took specific measures to ensure that the cells we recorded from were involved in the processing of chemonociceptive stimuli. The majority (26/38) of cells were of the wide-dynamic range type, responding to both noxious and non-noxious somatosensory stimuli with the rest being nociceptive-specific. All cells, however, responded robustly to pentanoic acid, a stimulus capable of activating the vanilloid receptor VR-1 (Tominaga *et al*., 1998). Indeed, we have used this stimulus in the past to identify Vc cells responsive to capsaicin (Dessirier *et al*., 2000b) and found a 100% correspondence to capsaicin sensitivity. Despite our attempts to target cells most likely to respond to a quinine stimulus, we saw no evidence of such activation. Indeed, we

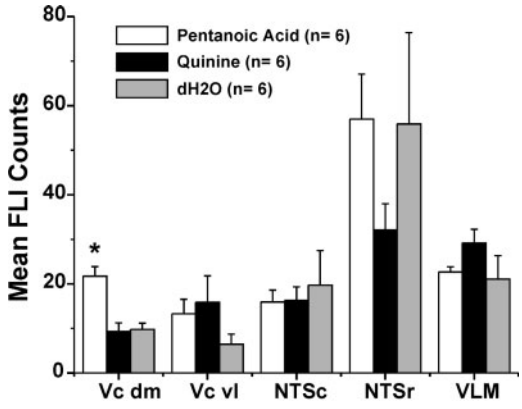

Figure 5 Bar graph of averaged (mean ± SEM) FLI counts for each indicated brainstem region elicited by pentanoic acid (white bars), quinine (black bars) and water (gray bars). Note that no significant differences exist in the number of FLI expressing neurons evoked by quinine and water in any of the brainstem regions examined whereas pentanoic acid elicited significantly higher FLI in Vc dm. Error bars: SEM. *Significantly different ($P < 0.001$) compared with quinine and dH_2O groups. Abbreviations: Vc dm, dorsomedial aspect of Vc; Vc vl, ventrolateral aspect of Vc; NTSc, caudal aspect of nucleus of the solitary tract from the level of the pyramidal decussation rostrally to the level of area postrema; NTSr, rostral NTS from the level of the AP to the rostral pole; VLM, ventrolateral medulla.

specifically attempted to identify transient, short-lasting responses, such as those reported earlier (Pittman and Contreras, 1998), but found no evidence for the occurrence of such activity in Vc. These findings were consistent with the c-fos results in which the number of FLI-expressing neurons evoked by quinine was not different from the number evoked following a water stimulus. Thus, whereas in some studies quinine evoked small and transient responses in thermally sensitive lingual fibers (Pittman and Contreras, 1998) or an increase in calcium in trigeminal ganglion cells (Liu and Simon, 1998), these effects do not appear to be translated as increased activity in Vc. There are several explanations that may account for this discrepancy. First, methodological and/or analytical variations may have contributed to the observed differences. Whereas we selected cells based upon chemonociceptive sensitivity, Pittman and Contreras (Pittman and Contreras, 1998) targeted thermally sensitive lingual fibers that responded to cooling. Moreover, their analysis was biased to specifically identify very subtle effects. Indeed, the criterion for a quinine-evoked response was a change in impulse frequency that need only exceed ±1.96 standard deviations of baseline activity. Secondly, although near the saturation limit for an aqueous solution, the concentration of quinine used presently may have been sub-threshold to activate chemonociceptive Vc neurons. We have noted on numerous occasions that the concentrations used to activate chemonociceptive Vc neurons far exceeds that needed to activate primary afferents from the cornea or induce changes in membrane permeability of cultured trigeminal ganglion cells (Carstens *et al*., 1998; Dessirier *et al*., 2000b; Simons *et al*., 2003). Third, the relatively small effects of quinine on lingual nerve fibers may be insufficient to excite Vc neurons that presumably receive considerable convergence from multiple trigeminal primary afferents. Lastly, the increased Ca^{2+} signal evoked by quinine in isolated trigeminal ganglion neurons (Liu and Simon, 1998) may reflect methodological anomalies such as thermal changes associated with stimulus delivery. Indeed, this increased calcium may not necessarily even have been sufficient to evoke action potentials in the afferent fiber. Alternatively, because quinine has been shown to alter membrane conductance in neuroblastoma × glioma hybrid cells (Robbins *et al*., 1992), the intracellular calcium increase reported in trigeminal ganglion cells may be the result of non-specific effects on membrane permeability.

Finally, it is of interest to note that presently, we found no evidence that quinine evoked greater FLI at the rostral pole of NTS than did water or pentanoic acid. In prior studies using awake, behaving animals, quinine was shown to evoke FLI along the medial aspect of the gustatory NTS suggesting the possibility of a rudimentary chemotopic map at this level of the central nervous system (Harrer and Travers, 1996; King *et al*., 1999; Travers *et al*., 1999; Travers, 2002). There are several explanations that might underlie this difference. The anesthetic (pentobarbital) used in the present experiments may well have blunted the neuronal taste responses to quinine, thus reducing the occurrence of FLI in the gustatory NTS. Moreover, in the present study, quinine was applied only to the anterior tongue thus exciting neurons predominantly of chorda tympani origin whereas in the awake behaving preparation, quinine is given through intraoral cannulae and thus activates gustatory neurons of chorda tympani as well as glossopharyngeal and vagal origin. Recent evidence suggests that the quinine-evoked FLI in the gustatory NTS is elicited primarily through glossopharyngeal afferents that terminate largely in the dorsomedial portion of this nucleus (King *et al*., 1999, 2000).

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