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Psychophysical and Neurobiological Evidence that the Oral Sensation Elicited by Carbonated Water is of Chemogenic Origin

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Abstract

The sensation produced by carbonated beverages has been attributed to chemical excitation of nociceptors in the oral cavity via the conversion of CO_2 to carbonic acid in a reaction catalyzed by carbonic anhydrase. In separate studies, we tested if the carbonic anyhdrase blocker, acetazolamide, reduced either the intensity of sensation in humans or c-fos expression by trigeminal neurons in rats, evoked by application of carbonated water to the tongue. In the psychophysical experiment, one-half of the dorsal tongue was pretreated with acetazolamide (1 or 2%), after which the tongue was exposed bilaterally to carbonated water. In a two-alternative forced-choice paradigm, subjects chose which side of the tongue yielded a stronger sensation and additionally rated the magnitude of sensation on each side. Pretreatment with acetazolamide reduced the magnitude of sensation elicited by carbonated water in a concentration-dependent manner, since a significant majority of subjects chose the untreated side of the tongue as having a stronger sensation and assigned significantly higher intensity ratings to that side. Acetazolamide did not affect the irritant sensation from citric acid, while capsaicin pretreatment reduced both the sensation elicited by carbonated water and the irritation induced by citric acid application. In a separate experiment using rats, delivery of carbonated water to the tongue significantly increased the number of cells expressing c-fos-like immunoreactivity in the dorsomedial trigeminal nucleus caudalis (versus saline controls); this was significantly reduced by pretreatment with acetazolamide. Our results support the hypothesis that carbonated water activates lingual nociceptors via conversion of CO_2 to carbonic acid; the nociceptors in turn excite trigeminal neurons involved in signaling oral irritation.

Introduction

The sensation elicited by carbonated beverages in the mouth is a pleasurable and sought-after sensation for many people despite the fact that this sensation is irritating or sometimes even painful. Indeed, the sensation associated with carbonation may be a major hedonic component contributing to the large consumption of carbonated drinks (Woodroof and Philips, 1981). It has been debated whether the sensation elicited by carbonated water is of mechanical origin due to bursting CO_2 bubbles stimulating mechanoreceptors or of chemogenic origin by the formation of carbonic acid which then stimulates polymodal nociceptors in the oral cavity. Several recent lines of evidence support the latter hypothesis. Of particular interest is the phenomenon of the 'champagne blues'. Mountaineers, after taking the carbonic anhydrase inhibitor acetazolamide (Diamox) for mountain sickness, reported that carbonated beverages lacked the tingle and beer tasted like 'dishwater' (Hannsson, 1961; Graber and Kelleher, 1988). Since carbonic anhydrase catalyzes the conversion of CO_2 to carbonic acid by the following reaction:

$$CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow HCO_3^- + H^+$$

these reports suggest that carbonic acid is necessary for the tingle of carbonated drinks. Additional support comes from a study in which the stinging/burning after-sensation of carbonated water persisted long after it had been expectorated (Green, 1992).

CO_2 gas at concentrations of >50% elicits pain when delivered to the eyes (Chen *et al.*, 1995) or nasal sinus (Cain and Murphy, 1980; Anton *et al.*, 1992), and solutions saturated with CO_2 elicit pain when infused into the skin (Steen *et al.*, 1992). CO_2 excites corneal nociceptors (Chen *et al.*, 1995, 1997), polymodal nociceptors in skin (Steen *et al.*, 1995), and lingual nerve (Komai and Bryant, 1993) and chorda tympani fibers (Kawamura and Adachi, 1967; Komai *et al.*, 1994) in a manner that is blocked by carbonic anhydrase inhibitors. Carbonated water also excites nociceptive neurons in trigeminal subnucleus caudalis (Carstens *et al.*, 1998). Therefore, current neurophysiological and psychophysical evidence supports the idea that CO_2 evokes

irritation by exciting trigeminal nociceptive processes via a carbonic anhydrase-dependent mechanism.

We tested this hypothesis in psychophysical experiments by assessing the effect of topical application of acetazolamide to the tongue on the perceived intensity of sensation evoked by carbonated water. To complement this, we also determined if carbonated water excites trigeminal nociceptive neurons in a carbonic anhydrase-dependent manner, as assessed by their expression of the immediate-early gene protein, c-fos, a marker of central neurons that are activated by a noxious stimulus (Hunt *et al.*, 1987; Zimmermann and Herdegen, 1994; Carstens *et al.*, 1995). In a study that appeared recently using a different stimulus application procedure, both the intensity of sensation and activation of rat trigeminal neurons were reduced by dorzolamide, a more lipid-soluble carbonic anhydrase blocker (Simons *et al.*, 1999). These latter results are consistent with the present findings using acetazolamide.

The popularity of carbonated drinks and spicy food has markedly increased in recent years, and both are frequently consumed together. We were therefore interested in investigating possible interactions between carbonated water and capsaicin, the pungent principle in chili peppers. It is well established that capsaicin can produce desensitization, that is, a reduction in sensations elicited by subsequent capsaicin and cross-desensitization to other irritant chemicals (Green, 1989, 1991; Dessirier *et al.*, 1997). We therefore investigated whether capsaicin might also exhibit cross-desensitization with the sensation elicited by carbonated water. This is important in establishing whether the sensation of carbonated water is mediated by capsaicin-sensitive fibers, and thus further supporting the chemogenic nature of this sensation. Portions of this study have appeared earlier in abstract format (Dessirier *et al.*, 1998).

Materials and methods

Experiment 1: psychophysical studies

Subjects

Twenty healthy non-smoking volunteers (seven males, 13 females, aged 18–34 years) who were students and staff members at the University of California at Davis participated in all experimental sessions unless otherwise stated. All fasted, except for water, for at least 1 h prior to each experimental session. Subjects were asked to refrain from eating spicy food for 2 days prior to the experimental session. The experiments were approved by the UC Davis Human Subjects review committee.

Chemical stimuli

The following chemicals were dissolved in distilled water (dH_2O) unless otherwise stated: acetazolamide (Sigma, St Louis, MO; 1% = 45 mM and 2% = 90 mM, pH 9.2), capsaicin (Sigma; 5 ppm = 16.4 μM from a 0.1% stock solution in 80% ethanol), citric acid (Fisher, Pittsburg, PA; 125 mM, pH 2.5), KCl (400 mM), NaOH (pH 9.2), quinine (0.4 mM with NaOH, pH 9.2), benzocaine (Oragel®, 20%; Del Pharmaceuticals Inc., Uniondale, NY) and carbonated water (6.57 g CO_2/l, Safeway, Pleasanton, CA).

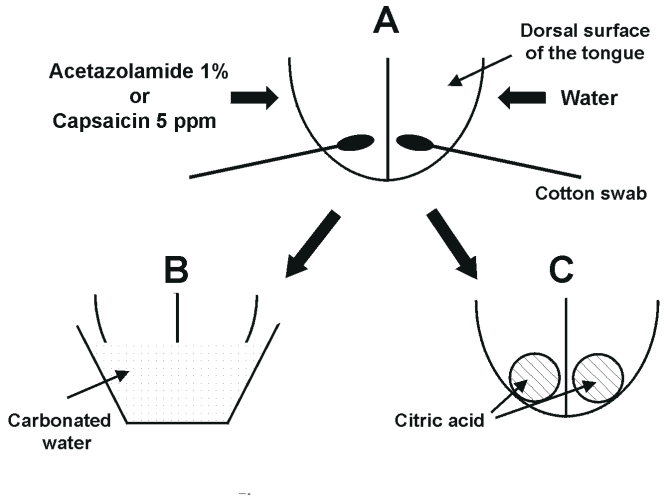

Figure 1 Psychophysical protocol. **(A)** In separate sessions, either acetazolamide or capsaicin was applied to one side of the tongue (hatching) and, simultanously, water was applied to the other side using cotton swabs. Two minutes after acetazolamide application, or 10 min after capsaicin application, subjects were then tested with either carbonated water (A) or citric acid (B). This gives a total of four tests, each performed in a separate session. **(B)** Carbonated water was tested by having the subject dip the tongue into a cup filled with freshly poured carbonated water. Subjects then indicated which side of the tongue yielded a stronger sensation (2-AFC), after which they separately rated the intensity of sensation on each side of the tongue using a category scale ranging from 0 (no sensation) to 10 (intense sensation). **(C)** Citric acid was tested by placing filter papers soaked with 40 μl of a 125 mM citric acid solution simultaneously onto each side of the tongue, after which subjects performed the 2-AFC and rating tests.

Experimental procedures

Acetazolamide effect on carbonation sensation. The effect of acetazolamide on the sensation produced by carbonated water was tested as follows. Acetazolamide (1%) was applied to one side of the anterior dorsal surface of the tongue, and dH_2O to the other, with cotton swabs (Figure 1A). Two minutes later subjects immersed the entire anterior tongue into a cup filled with carbonated water for up to 15 s (Figure 1B). Subjects then performed a two-alternative forced choice (2-AFC) test indicating which side of the tongue yielded a stronger sensation, followed by separate ratings of the intensity of sensation on the two sides of the tongue using a category scale ranging from 0 (no sensation) to 10 (very strong sensation). Data were analyzed using binomial and d' analyses (Ennis, 1993; Bi *et al.*, 1995) for the 2-AFC data and a *t*-test for the rating data with $P < 0.05$ considered significant.

To establish whether the effect of acetazolamide is concentration-dependent, the same experiment was conducted in an additional group of 25 subjects (nine males, 16

females, aged 18–48 years) using a higher (2%) acetazolamide concentration. In this case, acetazolamide was applied on one side of the tongue while a solution of 0.4 mM quinine, matched to acetazolamide pH (9.2) and taste intensity, was simultaneously applied to the other side, after which subjects rated the sensation elicited by carbonated water.

Acetazolamide effect on citric acid irritation. To control for non-specific effects of acetazolamide on proton-evoked irritation, the identical experiment was conducted using citric acid (40 µl; applied by filter papers onto both sides of the tongue; Figure 1C), rather than carbonated water, in a separate session.

Capsaicin cross-desensitization. We also tested if capsaicin cross-desensitized the sensations elicited by both carbonated water and citric acid. Capsaicin was sequentially applied to one side of the tongue with cotton swabs, five times successively at 1 min intervals; dH_2O was simultaneously applied in an identical manner on the other side (Figure 1A). After the last application the subject waited 10 min, followed either by immersion of the tongue into carbonated water (Figure 1B) or application of citric acid by filter paper (Figure 1C). To ensure that the capsaicin indeed had a desensitizing effect, 18 subjects were pretreated with capsaicin on one side of the tongue and then tested 10 min later with capsaicin applied to both sides of the tongue via filter papers as with citric acid.

pH control. To assess the possibility that the alkalinity of the acetazolamide solution affected the sensation elicited by carbonated water, a control experiment was performed on a separate group of 20 subjects (11 males, 9 females, aged 19–48 years) by applying dH_2O titrated with NaOH to a pH of 9.2, which matched that of the acetazolamide solution, on one side of the tongue and dH_2O at neutral pH on the other side. Subjects then dipped their tongue into the carbonated water and performed the 2-AFC and rating tests as above.

Tactile control. To control for possible local anesthetic effects of acetazolamide or capsaicin, the tactile sensitivity of the tongue was measured as follows. A weak von Frey monofilament (Stoelting, bending force 0.045 or 0.229 mN) was applied in random order 30 times to the tongue (10 stimuli to the side treated with acetazolamide or capsaicin, 10 to the non-treated side and 10 blanks). Subjects were asked to close their eyes and were prompted by a tone indicating impending stimulation, after which they indicated if they perceived whether a stimulus had been applied or not, and if they were sure or not sure of their judgement. From the response matrix an index of sensitivity (*R*-index) (O'Mahony, 1992) was calculated for individual subjects on each side of the tongue separately to determine if detection was poorer on the treated side. Responses were also pooled in a single matrix to compute an *R*-index across the entire subject population. Significance of *R*-indices was determined using tables (Bi and O'Mahony, 1995).

dH_2O control. To ensure that subjects did not exhibit asymmetry in the sensitivity of the two sides of the tongue (Merskey and Watson, 1979), both sides of the tongue were pre-treated with dH_2O followed by immersion of the whole tongue into carbonated water or bilateral stimulation with citric acid. The 2-AFC and intensity ratings were then performed as above.

Taste control. To control for the possibility that the slight bitter taste of acetazolamide and the obviously irritating sensation elicited by capsaicin might affect 2-AFC and intensity ratings, a taste control was performed. For this, KCl (400 mM; approximately matching acetazolamide taste intensity) was applied to one side of the tongue and dH_2O was applied to the other. Two minutes later the subjects rated carbonated water- and citric acid-elicited sensations.

Local anesthetic control. To verify the sensitivity of the 2-AFC and rating methods for the present experiments, we performed a control experiment at the onset of the study using the local anesthetic, benzocaine (20%; Oragel®). The rationale was that benzocaine should reduce tactile as well as chemically evoked sensations on the treated side of the tongue, and that subjects would choose the non-treated side as yielding stronger (or detectable) sensations and assign higher ratings (or probability of detection) to that side. The benzocaine was applied to one side of the tongue and dH_2O to the other side simultaneously. Sensations elicited by carbonated water, citric acid and tactile stimuli were assessed as described above.

Experiment 2: c-fos immunohistochemistry

Experiments were performed using adult male Sprague–Dawley rats (400–460 g body wt). The experimental protocol was approved by the UC Davis Animal Use and Care Advisory committee. Rats were anesthetized with sodium pentobarbital (65 mg/kg i.p.) and maintained under deep anesthesia for the entire 2 h experimental period with supplemental i.p. injections as needed. The mouth was held in a partly opened position with a small retractor, and a strip of Parafilm was placed underneath the tongue to prevent inadvertent stimulation of other oral tissue. One group of animals received topical application of isotonic NaCl (0.9%; 0.1 ml by syringe) followed by carbonated water (n = 6 rats). A second group (n = 6) received topical application of acetazolamide (1%; 0.1 ml) followed by carbonated water. A syringe pump was used to deliver 50 ml of carbonated water over a period of 10 min onto the anterior dorsal surface of the tongue. Twenty minutes after application of the carbonated water, the retractor was removed and the mouth closed to prevent desiccation. A third control group (n = 5) received topical application of

0.9% NaCl only, and a fourth control group ($n = 6$) did not receive any oral stimulation.

Two hours following application of carbonated water or other chemicals, the animals were perfused with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde through the aorta. Brainstems were removed and post-fixed for 8 h, and then transferred to a 30% sucrose solution. Two to three days later, the brainstems were cut in 50 µm frozen sections. Serial sections were successively collected in three separate 24-well containers (Costar, Corning, NY) in PBS, such that each container contained every third section at a 150 µm sampling interval. One container was then processed immunohistochemically. This involved washing the sections, blocking them in 3% normal goat serum and then incubating them in primary c-fos antibody (Arnel, New York; 1:50 000) for 24–36 h. The sections were then washed and exposed to secondary biotinylated (goat anti-rabbit) antibody, followed by an avidin–biotin–peroxidase complex (Vector, Burlingame, CA) reaction enhanced with biotinyl tyramide/H_2O_2. To visualize the reaction product, sections were finally subjected to a nickel-enhanced diaminobenzidine reaction. Sections were mounted on microscope slides and coverslipped. They were then examined under a light microscope (Nikon E-400) and cell nuclei displaying black fos-like immunoreactivity (FLI) were counted bilaterally in five regions of interest: the dorsomedial trigeminal nucleus caudalis (Vc), ventrolateral Vc, ventrolateral medullary area dorsal to the lateral reticular nucleus, nucleus of the solitary tract (NTS) and area postrema (Carstens *et al.*, 1995). Counts of FLI were made in all sections from the level of the pyramidal decussation caudally to the rostral extent of the area postrema. The investigator who did counts of FLI was blinded as to the experimental treatment. Between-treatment group comparisons of mean bilateral counts of FLI for each region of interest were statistically analyzed by an unpaired *t*-test, with $P < 0.05$ considered to be significant. Representative sections were imaged with a color video camera (Dage MTI DC–330) using Scion Image software, and imported to commercially available graphic software (CorelDraw) which allowed the locations of FLI to be plotted directly and accurately onto computer-generated traces of sections taken from the atlas of Paxinos and Watson (Paxinos and Watson, 1998).

Results

Experiment 1: psychophysical studies

Acetazolamide reduction of sensation elicited by carbonated water

When subjects received carbonated water following pretreatment with 1% acetazolamide to one side of the tongue, a significant majority (17/20, binomial, $P = 0.003$) chose the non-treated side as yielding a stronger sensation corresponding to a significant group d' value of 1.47 ($P = 0.02$) (Ennis, 1993; Bi *et al.*, 1995). Also, the mean intensity ratings for the treated and non-treated sides were significantly different (4.5 versus 5.5; *t*-test, $P = 0.01$; Figure 2A).

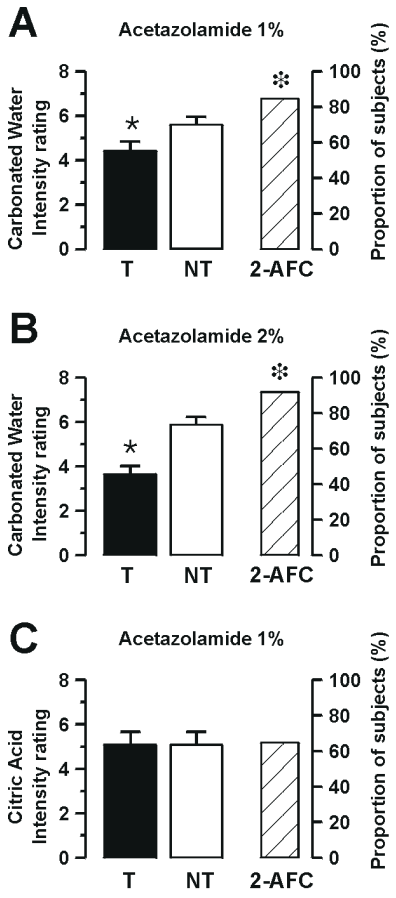

Figure 2 Psychophysical results: acetazolamide. In each panel, the pair of bars to the left indicate intensity ratings on the treated (T, filled bar) and non-treated (NT, open bar) side of the tongue, respectively. The hatched bar to the right indicates the proportion of subjects who chose the non-treated side to yield a stronger sensation in the 2-AFC test. **(A)** Effect of acetazolamide (1%) pretreatment on irritation evoked by carbonated water. **(B)** Effect of acetazolamide (2%) pretreatment on irritation evoked by carbonated water. **(C)** Effect of acetazolamide (1%) pretreatment on irritation evoked by citric acid. Error bars: SE. Asterisks over filled bars indicate significant difference between T and NT ($P < 0.05$, *t*-test). Stars over hatched bars indicate that a significant majority of subjects chose non-treated side ($P < 0.05$, binomial test).

Following pretreatment with 2% acetazolamide, a significant majority of subjects chose the non-treated side as having a stronger sensation (23/25 subjects; binomial, $P < 0.001$), yielding a group d' value of 1.99 ($P < 0.001$), and the mean intensity rating on the treated and non-treated sides were significantly different (3.7 versus 5.7; *t*-test, $P < 0.001$; Figure 2B). In addition, the difference in intensity ratings between the treated and non-treated sides was significantly greater after treatment with 2% acetazolamide compared with 1% acetazolamide (*t*-test, $P = 0.049$; Figure 2A,B). Thus, the results indicate that acetazolamide reduced the

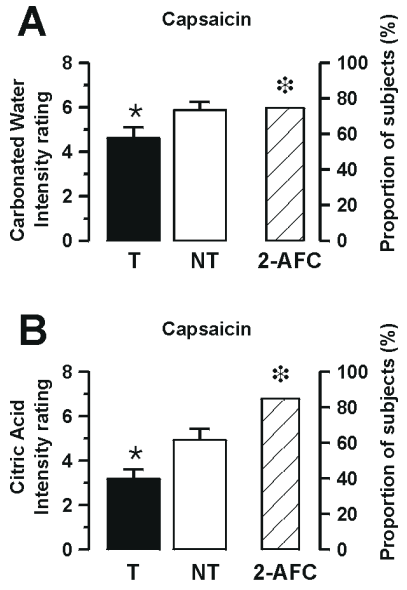

Figure 3 Psychophysical results: capsaicin. **(A)** Effect of capsaicin pretreatment on irritation evoked by carbonated water. **(B)** Effect of capsaicin pretreatment on irritation evoked by citric acid. Format as in Figure 2.

sensation elicited by carbonated water on the tongue in a dose-related manner.

Lack of effect of acetazolamide on citric acid-evoked irritation

Acetazolamide had no effect on the irritation induced by citric acid, judging from a lack of significance in both 2-AFC (13/20 subjects; binomial, $P = 0.26$) and mean ratings between the treated and non-treated sides (5.1 on both sides; *t*-test, $P = 1$; Figure 2C).

Cross-desensitization effects of capsaicin

Pretreatment of the tongue with capsaicin led to a cross-desensitization of the sensations produced by subsequent application of both carbonated water and citric acid. Thus, a significant majority of subjects chose the non-treated side as yielding a stronger sensation (significant group d' values of 0.96, $P = 0.026$, for carbonation and 1.47, $P = 0.02$, for citric acid) and assigned significantly higher ratings to that side following application of both carbonated water (Figure 3A) and citric acid (Figure 3B). This apparent cross-desensitizing effect of citric acid irritation by capsaicin confirms earlier results (Gilmore and Green, 1993). Capsaicin self-desensitization was verified, also confirming previous reports (Green, 1989; Dessirier *et al.*, 1997). Thus, 18/18 subjects chose the capsaicin non-pretreated side to yield stronger irritation (Binomial, $P < 0.001$), and ratings were significantly higher on that side (5.5 versus 3.11; *t*-test, $P < 0.001$).

pH control

Treatment of one side of the tongue with a control solution at pH 9.2 did not have any effect on the sensation elicited by carbonated water (11/20 subjects; binomial, $P = 0.82$; intensity ratings of 4.3 versus 4.3; *t*-test, $P = 0.87$), arguing against any non-specific inhibitory effects due to the alkalinity of the acetazolamide solution.

Tactile control

Neither acetazolamide nor capsaicin had any effect on tactile sensitivity. *R*-index measures indicated no significant difference in detection of tactile stimuli between the treated and non-treated sides of the tongue. The pooled *R*-indices representing the average tactile sensitivity on each side of the tongue were not significantly different (acetazolamide: 90.6 versus 86.9, $P = 0.17$; capsaicin: 85.2 versus 89.1, $P = 0.17$). Moreover, no difference in tactile sensitivity was observed when dH_2O was applied on both sides of the tongue or when KCl was applied on one side of the tongue, thus demonstrating that there was no asymmetry in tactile sensitivity between the two sides of the tongue.

Local anesthetic control

The *R*-index method successfully detected a decrease in tactile sensitivity caused by pre-treatment with benzocaine, further verifying the sensitivity of the present half-tongue method. Thus, the mean *R*-index was significantly decreased on the benzocaine-treated side (70.4 versus 82.5; *t*-test, $P < 0.001$) in a significant majority of subjects (17/20; binomial, $P < 0.003$). Also, the value of the pooled *R*-index representing the difference in sensitivity between the two sides of the tongue was significantly greater than 50% (66.6, $P < 0.001$).

Benzocaine also reduced sensitivity to citric acid irritation. Thus, when citric acid was applied bilaterally following unilateral pretreatment with benzocaine, subjects reliably chose the non-treated side as yielding a stronger irritation (17/20; binomial, $P = 0.003$) and the mean ratings on this side were significantly higher (5.0 versus 3.0; *t*-test, $P = 0.003$). Interestingly, benzocaine had a weaker effect on irritation from carbonated water. Both the percentage of subjects choosing the non-treated side as stronger (14/20, binomial, $P = 0.11$) and the difference in mean intensity ratings between the treated and non-treated sides (4.5 versus 3.3; *t*-test, $P = 0.09$) failed to reach statistical significance.

Taste and dH_2O controls

Finally, there was no significant lateral bias, or difference in ratings, following unilateral treatment with KCl (carbonation: 12/20 subjects, binomial, $P = 0.5$; 5.0 versus 4.6, *t*-test, $P = 0.3$; citric acid: 12/20 subjects, binomial, $P = 0.26$; 4.1 versus 4.0, *t*-test, $P = 0.82$) or bilateral treatment with dH_2O (carbonated water: 11/20 subjects, binomial, $P = 0.82$; 5.2 versus 5.4, *t*-test, $P = 0.72$; citric acid: 10/20 subjects, binomial, $P > 0.82$; 5.5 versus 5.3, *t*-test, $P = 0.72$), arguing against any taste effects or consistent asymmetry in chemical sensitivity of the two sides of the tongue.

Experiment 2: c-fos immunohistochemistry

Following application of carbonated water to the tongue,

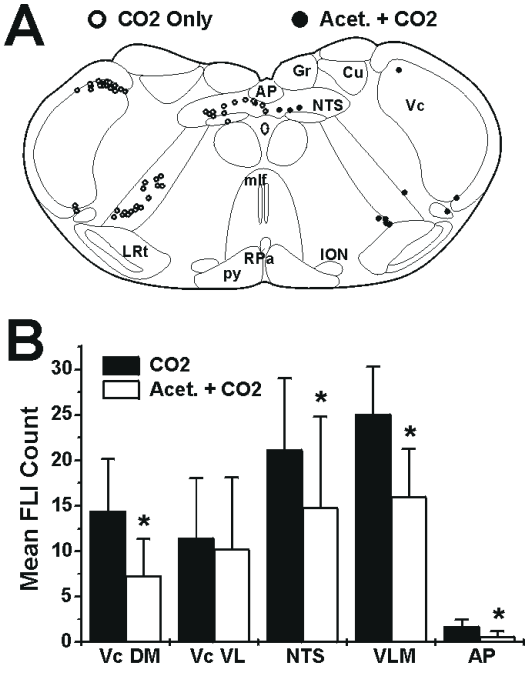

Figure 4 Brainstem distribution of FLI following application of carbonated water, and reduction by acetazolamide. **(A)** Cross-section of brainstem [anterior –5.08; from the atlas of Paxinos and Watson (Paxinos and Watson, 1998)]. Dots indicate locations of cells expressing FLI. The left half of section shows the distribution of FLI from animal receiving carbonated water only. The right half shows the distribution of FLI from an animal receiving acetazolamide (1% to lingual surface) followed by carbonated water. Abbreviations: AP, area postrema; Cu, n. cuneatus; Gr, n. gracilis; ION, inferior olivary n.; LRt, lateral reticular n.; mlf, medial longitudinal fasciculus; NTS, n. of solitary tract; py, medullary pyramid; RPa, n. raphe pallidus; Vc, trigeminal subnucleus caudalis. **(B)** Bar graph plotting mean counts of FLI in animals receiving carbonated water only (CO_2; shaded bars; $n = 6$), and carbonated water after pretreatment with acetazolamide (Acet. + CO_2; open bars; $n = 6$). Each pair of bars shows the mean counts of FLI for the five different regions indicated. Abbreviations: Vc DM, dorsomedial aspect of trigeminal caudalis; VL Vc, ventrolateral aspect of trigeminal caudalis; NTS = n. of solitary tract; VLM = ventrolateral medulla; AP = area postrema. Error bars: SEM. *Significantly different from CO_2 only ($P < 0.05$, unpaired *t*-test).

the number of cells expressing FLI was significantly higher ($P < 0.05$, unpaired *t*-test) in the dorsomedial and ventrolateral aspects of Vc compared with saline-treated controls, and was significantly higher for all regions compared with unstimulated controls. The left half of the section in Figure 4A shows an example of the distribution of FLI in an animal that received carbonated water only. Note the presence of FLI within dorsomedial and ventrolateral Vc, NTS, and ventrolateral medulla. The right half of the section in Figure 4A shows FLI in an animal that received carbonated water after pretreatment with acetazolamide, and illustrates a reduction in FLI in dorsomedial Vc and other regions in comparison with animals receiving carbonated water alone. These results are similar to our findings using a different carbonic anhydrase blocker, dorzolamide, as described in more detail elsewhere (Simons *et al.*, 1999). Mean counts of FLI are shown graphically in Figure 4B. Pretreatment with acetazolamide significantly reduced counts of FLI in the dorsomedial Vc, NTS, ventrolateral medulla and area postrema.

Discussion

One of the main findings of this study is that the carbonic anhydrase inhibitor acetazolamide reduced the sensation induced by carbonated water, but not that induced by citric acid, on the tongue. In complementary animal experiments, acetazolamide significantly reduced FLI in the dorsomedial Vc elicited by application of carbonated water to the tongue.These data support the idea that the sensation elicited by carbonated water on the tongue requires conversion of CO_2 into carbonic acid to stimulate chemosensitive fibers within the lingual epithelium, whose afferent fibers in turn project to Vc to excite neurons there. This idea is further supported by another study from our laboratory showing that a different carbonic anhydrase inhibitor, dorzolamide, reduced the intensity of sensation in humans, as well as both c-fos expression and electrophysiologically recorded responses of single neurons in Vc of rats elicited by carbonated water (Simons *et al.*, 1999). The present findings confirm earlier animal studies showing inhibition of lingual nerve and chorda tympani fiber responses to carbonated water by carbonic anhydrase inhibitors (Komai and Bryant, 1993; Komai *et al.*, 1994).

Another main finding of the present study was that pretreatment of the tongue with capsaicin reduced irritant sensations elicited by subsequent application of carbonated water or citric acid (cross-desensitization), the latter finding confirming an earlier report (Gilmore and Green, 1993). The means by which capsaicin does this is not certain. This process appears to require binding of capsaicin to a 'vanilloid' (VR-1; capsaicin) molecular receptor within the terminal membrane of nociceptors (Caterina *et al.*, 1997), which in turn leads to a persistent reduction in chemical excitability of the nociceptor due partly to calcium influx (Cholewinski *et al.*, 1993; Chard *et al.*, 1995; Liu and Simon, 1996; Caterina *et al.*, 1997). Our present findings suggest that the irritation produced by CO_2 and citric acid is conveyed at least partly by capsaicin-sensitive nerve fibers. Indeed, some nerve fibers can be excited by both capsaicin and acid or CO_2 (Belmonte *et al.*, 1991; Chen *et al.*, 1997). Also, the capsaicin-induced cross-desensitization of the sensation elicited by carbonated water was weaker than that observed for citric acid irritation, suggesting that a smaller proportion of capsaicin-sensitive fibers are activated by carbonated water than by citric acid. Indeed, only 50% of the corneal cells activated by CO_2 gas also responded to capsaicin (Chen *et al.*, 1997). Another possibility to explain the present results is that CO_2, being a small lipid-soluble molecule, diffuses further within the lingual epithelium to reach nerve endings that were not desensitized by capsaicin.

Various control experiments were conducted to rule out the possibility that the psychophysical results were due to an inhibition of the tactile sensation produced by bubbles bursting on the tongue, to a bias resulting from the taste of acetazolamide or to a non-specific effect of the alkalinity of the acetazolamide solution. Application of a local anesthetic agent to the tongue also significantly reduced tactile sensitivity, showing that the method used to assess changes in mechanical sensitivity was able to pick up differences between the two sides of the tongue (positive control). Application of the local anesthetic also inhibited the irritation produced by topical application of citric acid, which confirmed the ability of the half-tongue method to detect differences. A possible explanation for why the local anesthetic did not strongly reduce the sensation elicited by carbonated water is, as for the weak effect of capsaicin desensitization, that CO_2 penetrates more deeply into the lingual epithelium than protons originating from acids that have dissociated at the lingual surface. CO_2 might therefore activate polymodal nociceptors in deeper tissue layers that were not reached by the local anesthetic applied topically.

It is currently not known if protons from acids, and carbonic acid from CO_2, excite nociceptors via a common cellular mechanism once they reach the site(s) of stimulus transduction. A possible extracellular transduction mechanism may involve one or more of the acid-sensitive ion channels that have been recently identified (e.g. acid-sensing ion channel = ASIC; dorsal root acid-sensing ion channel = DRASIC) (Waldmann, *et al.*, 1997a,b; Chen *et al.*, 1998). However, a substantial fraction of lingual nerve fibers was shown to respond to carbonated water but not acid at even lower pH (Komai and Bryant, 1993), suggesting that mucosal pH is not the only determinant of CO_2 stimulation. As an alternative to an extracellular site of action of carbonic acid, CO_2 might enter into the nociceptor fiber terminals in the tongue, where carbonic anhydrase catalyzes its conversion into carbonic acid to result in intracellular acidification.

Interestingly, sensory intensity ratings were only partially reduced by pretreatment with acetazolamide. The residual perceived sensation following acetazolamide pretreatment might have been evoked by a mechanical or osmotic component of the carbonated water stimulus. Our present results suggest that acetazolamide specifically reduces the activation of lingual nociceptors by carbonated water to result in a reduction in irritation but not other potential components of the resultant sensation.

The immunohistochemical data, showing that CO_2-evoked FLI in the dorsomedial Vc was significantly reduced by acetazolamide, are consistent with the present psychophysical results as well as results from our study using dorzolamide (Simons *et al.*, 1999). These findings support the idea that carbonated water causes a carbonic anhydrase-dependent excitation of lingual nociceptors which, in turn, excite neurons in Vc that mediate the sensory transmission of oral irritation. We previously reported that neurons in the dorsomedial Vc, as well as in other brainstem regions, including the ventrolateral Vc, NTS, ventrolateral medulla and area postrema, showed significant increases in FLI following application of various irritant chemicals to the tongue, including capsaicin, nicotine, piperine and histamine (Carstens *et al.*, 1995). In the present study, carbonated water also produced a significant increase in FLI in the ventrolateral Vc versus saline controls which, however, was not carbonic anhydrase-dependent. Conceivably, FLI might have been evoked by mechanical or osmotic components of the stimulus (see above). Possible roles for ventrolateral Vc neurons in oral irritation and other functions have been suggested previously (Strassman and Vos, 1993; Carstens *et al.*, 1995). Acetazolamide pretreatment also reduced FLI in the NTS, ventrolateral medulla and area postrema (Figure 4B). While there are no previous data regarding possible roles for these regions in oral sensory processing (see Carstens *et al.*, 1995), the present data suggest that neurons in these areas may also be activated by oral application of carbonated water in a carbonic anhydrase-dependent manner.

In conclusion, the present study provides psychophysical and neuroanatomical support for the hypothesis that the sensation elicited by carbonated drinks is at least partly of chemogenic origin. Dissolved CO_2 is converted via carbonic anhydrase into carbonic acid, which excites intraoral nociceptors that, in turn, activate neurons in the Vc to signal sensations of oral irritation.

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