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Neurobiological and Psychophysical Mechanisms Underlying the Oral Sensation Produced by Carbonated Water

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Carbonated drinks elicit a sensation that is highly sought after, yet the underlying neural mechanisms are ill-defined. We hypothesize that CO_2 is converted via carbonic anhydrase into carbonic acid, which excites lingual nociceptors that project to the trigeminal nuclei. We investigated this hypothesis using three methodological approaches. Electrophysiological methods were used to record responses of single units located in superficial laminae of the dorsomedial aspect of trigeminal subnucleus caudalis (Vc) evoked by lingual application of carbonated water in anesthetized rats. After pretreatment of the tongue with the carbonic anhydrase inhibitor dorzolamide, neuronal responses to carbonated water were significantly attenuated, followed by recovery. Using c-Fos immunohistochemistry, we investigated the distribution of brainstem neurons activated by intraoral carbonated water. Fos-like immunoreactivity (FLI) was significantly higher in the superficial laminae of dorsomedial and ventrolateral Vc in animals treated with carbonated water versus controls. Dorzolamide pretreatment significantly reduced FLI in dorsomedial Vc. We also examined the sensation elicited by carbonated water in human psychophysical studies. When one side of the tongue was pretreated with dorzolamide, followed by bilateral application of carbonated water, a significant majority of subjects chose the untreated side as having a stronger sensation and assigned significantly higher intensity ratings to that side. Dorzolamide did not reduce irritation elicited by pentanoic acid. The present data support the hypothesis that carbonated water excites lingual nociceptors via a carbonic anhydrase-dependent process, in turn exciting neurons in Vc that are presumably involved in signaling oral irritant sensations.

Key words: trigeminal nucleus caudalis; c-Fos; single-unit recording; rat; carbonated water; carbonic anhydrase; oral irritation; psychophysics; two-alternative forced-choice

The allure of carbonated beverages is supported by the $55 billion generated in retail sales in 1997 (Beverage Digest, 1998). Despite this huge fiscal impact, relatively little is known about the neural mechanisms underlying the sensation elicited by carbonation. Carbon dioxide applied to the corneal surface (Chen et al., 1997), nasal epithelium (Cain and Murphy, 1980; Anton et al., 1991a,b, 1992; Thürauf et al., 1991, 1993; Peppel and Anton, 1993), or skin (Steen et al., 1992) excites nociceptive fibers and evokes pain sensation in humans. CO_2 interacts with water in a reaction catalyzed by carbonic anhydrase to form carbonic acid, which presumably stimulates chemosensitive nociceptors (Lingueglia et al., 1997; Waldmann et al., 1997a,b). Indeed, carbonic anhydrase inhibitors attenuate the activity elicited by saturated CO_2 solutions in cutaneous (Steen et al., 1992) and chorda tympani nerve fibers (Kawamura and Adachi, 1967; Komai et al., 1994).

It has been debated whether the oral sensation produced by carbonated beverages is primarily chemogenic or rather mechanical in nature because of bursting CO_2 bubbles (Yau and McDaniel, 1990, 1991; Green, 1992; Komai and Bryant, 1993). Several lines of evidence argue against the mechanical hypothesis. Tingling, mouth-burn, pricking, and other sensations elicited by carbonated water under normal atmospheric conditions were essentially unchanged when subjects ingested the carbonated water under hyperbaric conditions (3.4 atmosphere) in which bubble formation was prevented (McEvoy, 1998). Furthermore, subjects consistently chose poignant over tactile descriptors in describing the sensation elicited by carbonated water and reported a "burning and tingling–numbness" aftersensation long after the carbonated water had been expectorated (Green, 1992). Finally, the carbonic anhydrase blocker acetazolamide was reported to reduce the "fizziness" of carbonated drinks (Graber and Kelleher, 1988) and the response of lingual nerve nociceptive afferents to carbonated water (Komai and Bryant, 1993).

The present study sought to further characterize the mechanisms underlying the oral sensation elicited by carbonated water using three distinct methodologies. The first involved single-unit recordings from trigeminal subnucleus caudalis (Vc). Primary afferent fibers of nociceptors originating in the orofacial region project to Vc (Hayashi, 1985; Jacquin et al., 1986; Komai and Bryant, 1993; Coimbra and Coimbra, 1994; Strassman and Vos, 1993), as well as other trigeminal subnuclei (see Discussion), in which they activate second-order neurons presumably involved with relaying nociceptive information to higher centers (Kruger and Michel, 1962; Yokota, 1975; Amano et al., 1986; Strassman and Vos, 1993; Carstens et al., 1995, 1998; Raboisson et al., 1995). In particular, neurons in superficial laminae of the dorsomedial aspect of Vc are activated by application of irritant chemicals to the tongue (Carstens et al., 1995, 1998). We tested the hypothesis that carbonated water excites neurons in Vc by activating intraoral nociceptors via a carbonic anhydrase-dependent mechanism.

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Lingual application of a variety of irritant chemicals resulted in a similar distribution of c-Fos expression in several brainstem regions, including dorsomedial Vc, ventrolateral Vc, nucleus of the solitary tract (NTS), area postrema (AP), and ventrolateral medulla (Carstens et al., 1995). Carbonated water might conceivably activate neurons in these and/or other brainstem areas. Moreover, defining which brainstem nuclei are activated by CO_2 may shed some light on its interaction with other irritants and tastants (Cometto-Muñiz et al., 1987; Yau and McDaniel, 1992; Cowart, 1998). We therefore used the method of c-Fos immunohistochemistry to investigate the brainstem distributions of neurons activated by carbonated water and whether it is prevented by pretreatment with a carbonic anhydrase inhibitor dorzolamide.

Finally, to provide a perceptual correlate for the neurobiological findings, we conducted human psychophysical studies to determine whether dorzolamide pretreatment selectively reduces the sensation elicited by carbonated water but not other acids.

MATERIALS AND METHODS

Experiment 1: electrophysiology

Animals. Ten adult male Sprague Dawley rats (Simonsen Inc., Gilroy, CA), weighing between 380–480 gm, were used in the experiments. The animals were housed one per cage in a room maintained on a 12 hr light/dark cycle and an ambient temperature of 21 ± 2°C. Food and water were available *ad libitum*. To obviate any possible effects of circadian rhythms (Lotsch et al., 1998), the experiments were always started between 12:00 P.M. and 2:00 P.M. All protocols were approved by the University of California (UC) Davis Animal Use and Care Advisory committee.

Surgery. Each animal was anesthetized with thiopental (80 mg/kg, i.p.). Core body temperature was maintained at ~37°C by placing the animal on a heating pad. A tracheotomy was performed, and a tracheal cannula was implanted. Similarly, a catheter was inserted into the jugular vein so thiopental could be infused intravenously ($10\ mg \cdot kg^{-1} \cdot d^{-1}$) during the course of the experiment to maintain anesthesia; the rate was increased briefly if the rat showed signs of insufficient anesthesia, such as change in heart rate or reflexive movement in response to a noxious stimulus. The occipital bone and upper cervical spine were visualized via a midline incision, and the base of the cerebellum, lower brainstem, and C1 spinal cord were exposed by removal of the caudal portion of the occipital bone and atlas. Each animal was then placed in a stereotaxic frame (Kopf Instruments, Tujunga, CA) with the head slightly ventroflexed and the upper cervical spine immobilized with a vertebral clamp. The dura mater was removed, and an agar (Difco, Detroit, MI) pool was formed over the brainstem. After the agar hardened, an opening was cut in an area overlying the target recording site and filled with 0.9% saline. Finally, a small clip was placed over the upper and lower incisors in such a way as to keep the mouth open and the tongue easily accessible. Isotonic saline was applied frequently to the tongue to prevent desiccation.

Recording. A Teflon-insulated tungsten recording microelectrode (~10 MΩ; F. Haer Inc., Brunswick, ME) was advanced into the brainstem in 5 μm steps using a hydraulic microdrive (Kopf Instruments). Extracellular single-unit activity was amplified and displayed by conventional means and fed via an analog-to-digital converter (Microstar Industries, Seattle, WA) to a computer for analysis and storage. Unitary action potentials were discriminated, counted, and displayed in peristimulus-time histogram (PSTH) format (bin width of 1 sec), using software developed in Erlangen, Germany (Forster and Handwerker, 1990).

Recordings were made from single units in the superficial layers of the dorsomedial aspect of Vc that responded to ipsilateral mechanical (touch, pressure, pinch) and heat (~54°C) stimuli, and carbonated water was applied bilaterally to the dorsoanterior one-third of the tongue. The search for units was restricted to an area previously shown to contain neurons responsive to noxious chemical stimulation of the tongue (Carstens et al., 1995, 1998). Briefly, this region included the area approximately between 0.5 mm rostral and 2 mm caudal to the obex, and 1.5 mm lateral. Tongue units responsive to mechanical stimulation were readily observed at depths ranging from 50 to 500 μm below the medullary surface.

Stimulation. The stimuli used to induce activity in trigeminal neurons included noxious heat [hot (~ 54°C) deionized water], buffered hydrochloric acid, pH 1 (Fisher Scientific, Pittsburgh, PA), and commercially available carbonated water, pH 3.4 ± 0.1 (Safeway Foods, Inc., Pleasanton, CA).

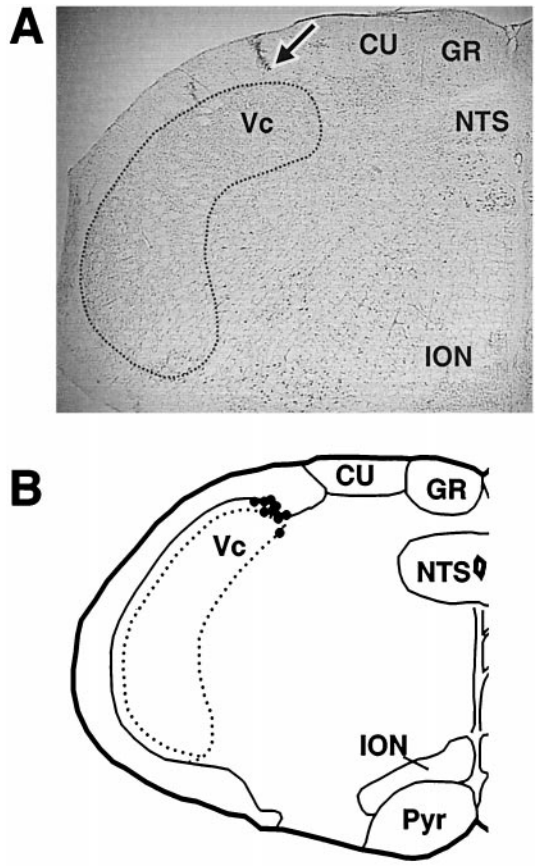

Figure 1. Histologically recovered recording sites in superficial laminae of the dorsomedial Vc. *A*, Photomicrograph of lesion site in Vc (*arrow*). *B*, Recording sites (●) for nine units, compiled on representative section of brainstem. Lateral *dashed lines* indicate approximate border of laminae I–II, and medial *dashed lines* indicate approximate ventral border of Vc. *CU*, Cuneate nucleus; *GR*, nucleus gracilis; *ION*, inferior olivary nucleus; *Pyr*, pyramid.

A syringe was used to apply stimuli bilaterally to the anterodorsal surface of the tongue in ~0.1 ml volume (except carbonated water, which was applied continuously at the rate of ~0.1 ml/sec). All chemicals were delivered at room temperature to avoid any confounding effects of cooling or heating. The acid stimulus was applied as a bolus, left on for 30 sec, and then immediately rinsed with ~2 ml of isotonic saline. Freshly opened carbonated water was applied continuously for 30 sec, followed immediately by a saline rinse.

In cells showing stable responses to carbonated water applied three times in succession at 5 min interstimulus intervals, the carbonic anhydrase inhibitor dorzolamide hydrochloride (22.3 mg/ml; Merck, West Point, PA) was then delivered. Dorzolamide was applied three times (at 0, 5, and 10 min) as a bolus (~0.1 ml) to the dorsal lingual epithelium. After the last application, the drug was left on the lingual surface for 10 min, after which the response of the unit to carbonated water was recorded again. If the response of the cells to carbonated water was reduced after dorzolamide, we continued testing with carbonated water at ~10 min intervals to determine whether the response recovered to predorzolamide levels.

Histology. After the completion of each recording session, an electrolytic lesion was made at the recording site by passing current (6 V DC) through the microelectrode for 20 sec. Animals were then killed with a lethal overdose of thiopental (intravenously), and the brains were removed and post-fixed in 10% formalin. The brainstems were frozen, cut into 50 μm sections, collected on glass slides, counterstained with neutral red, and examined under a light microscope. Lesions were identified and collectively plotted onto a representative brainstem section (Fig. 1).

Data analysis. Three conditions were used in these experiments (control, control plus dorzolamide, and recovery). Data from each unit under each condition were integrated over the initial 15 sec [spontaneous activity before carbonated water application (0–15 sec)] and again over the next 15 sec period (16–31 sec) during the initial period of carbonated water application and before any significant adaptation occurred. The effects of dorzolamide treatment were analyzed across all units using a Student's paired *t* test to compare responses to carbonated water before and after dorzolamide application; $p < 0.05$ was taken to be significant.

Experiment 2: immunohistochemistry

Animals. Twenty-four adult male Sprague Dawley rats (Simonsen Inc.), weighing between 400–500 gm, were used in the experiments. The animals were housed in the same manner as described above. Twenty-four hours before the experiment, the animals were brought to the laboratory and acclimated to the experimental environment. The experimental protocol was approved by the UC Davis Animal Use and Care Advisory committee.

Stimulation. Animals were anesthetized with sodium pentobarbital (65 mg/kg, i.p.). Once a proper plane of anesthesia was attained as assessed by areflexia, a clip was placed gently over the upper and lower incisors to hold the mouth open slightly. Parafilm (American National Can, Neenah, WI) was gently placed under the tongue to prevent stimuli from contacting the gingiva and other oral surfaces. Animals received the following chemical stimuli. (1) Carbonated water, pH 3.4 ± 0.1 (Safeway Foods Inc.), which was flowed onto the lingual surface at a rate of ~10 ml/min for 10 min ($n = 6$). (2) Dorzolamide hydrochloride (22.3 mg/ml; Merck), applied topically three times at 5 min intervals by bolus application (0.1 ml) to the dorsal surface of the tongue ($n = 11$). The third application was left on for 10 min, after which carbonated water was applied in the same manner as above. (3) Isotonic saline control, applied in the same manner as dorzolamide ($n = 5$). This control group allowed us to assess the degree of stimulation potentially caused by dorzolamide application. (4) A flat water control ($n = 7$). The flat water was made by exposing the same carbonated water as used previously to air for 24 hr and was assessed qualitatively by the investigators. The flat water was flowed at the same rate as the carbonated water; this group controlled for any mechanical activation of lingual fibers by the flow. (5) Unstimulated controls ($n = 6$). This last control group was perfused 2 hr after induction of anesthesia without any type of stimulation; this allowed us to determine basal levels of c-Fos expression. After the stimulation procedure, the incisor clip was carefully removed, and the animals were allowed to lie quietly on a heating pad until the perfusion.

Staining. Two hours after the onset of stimulation with carbonated water (or saline or flat water), the rats were perfused through the heart with 250 ml of PBS, followed by 500 ml of 4% paraformaldehyde. The brains were removed and post-fixed for 24–48 hr, after which they were placed in a 30% sucrose solution for cryoprotection. One to 2 d later, the brainstems were frozen, cut into 50 μm sections, and processed for c-Fos immunohistochemistry. The sections were first blocked with 3% normal goat serum (in PBS with 0.3% Triton X-100) for 1 hr and then exposed to the primary c-Fos antibody (diluted 1:50,000; Arnel Products Inc., New York, NY) for 24–36 hr. The primary antibody was removed, sections were washed, and the secondary biotinylated goat anti-rabbit antibody (Vector Laboratories, Burlingame, CA) was applied. One hour later, this antibody was removed, and the sections were washed again and then subjected to an avidin–biotin–peroxidase reaction (Vector Laboratories). Finally, cell nuclei expressing Fos-like immunoreactivity (FLI) were stained black by a nickel diaminobenzidine reaction. Brainstem sections were mounted on gelatin-coated slides, air-dried, cleared in alcohol, and coverslipped. The locations of cell nuclei expressing FLI were observed and quantified under the light microscope (E-400; Nikon, Tokyo, Japan).

Data analysis. The numbers of cell nuclei with FLI were counted in five areas of the brainstem shown previously to be responsive to irritant chemicals placed on the tongue (Carstens et al., 1995). Specifically, these areas were (1) the dorsomedial aspect of Vc, (2) the ventrolateral aspect of Vc, (3) the NTS between the level of the pyramidal decussation caudally and area postrema rostrally, (4) a region of the ventolateral medulla near the lateral reticular nucleus, and (5) the AP (see Fig. 5). Brainstem sections were selected for quantification of FLI so that comparisons with sections at corresponding levels of the brainstem could be made between animals. The investigator who selected sections and did the counts was blinded as to the experimental treatment. For illustrations, representative sections were imaged with a color video camera (DC-330; Dage-MTI, Michigan City, IN) using Scion Image software (Scion Corp., Frederick, MD) and imported to commercially available graphical software (Corel Draw; Corel, Ottawa, Ontario, Canada), which allowed locations of FLI to be plotted directly and accurately onto a computer-generated trace of the section. 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.

Experiment 3: human psychophysics

Subjects. Twenty-one subjects (10 male, 11 female) ranging in age from 19 to 29 years participated in the experiments; all were students at UC Davis. The protocol was approved by the UC Davis Human Subjects Review committee. Subjects were asked to refrain from eating, drinking, or smoking for at least 1 hr immediately before the experiment (three subjects reported to be smokers). Subjects participated in a single session that lasted <30 min.

Stimuli. The carbonic anhydrase inhibitor dorzolamide hydrochloride (22.3 mg/ml; Merck) was used at full strength. A control solution was prepared that approximately matched the dorzolamide in viscosity [1% methyl cellulose (Sigma, St. Louis, MO) in deionized water] and taste (1.26 mM quinine HCl; BDH Chemicals, Poole, UK). The control solution was carefully chosen so as to minimize bias and to equalize any bitter-induced inhibition of CO_2 pungency as described previously by Cometto-Muñiz et al. (1987). The carbonated water stimulus, pH 3.4 ± 0.1, was prepared in our laboratory by pressurizing (50 psi) deionized water at room temperature with CO_2 (95%) for 2 d. Pentanoic acid (Sigma) was diluted with deioinized water to a final concentration of 200 mM. To test lingual sensitivity to tactile stimulation, a von Frey monofilament (Stoelting, Chicago, IL) calibrated to 0.229 mN was used.

Stimulus application. The primary purpose of this experiment was to determine whether previous treatment with the carbonic anhydrase inhibitor dorzolamide attenuated the perceived sensation caused by the application of carbonated water to the human lingual surface. To this end, we used a half-tongue protocol similar to one used previously (Dessirier et al., 1997, 1998). Briefly, a filter paper disk (2.5 cm diameter; Whatman International Ltd., Maidstone, UK) was cut in half, saturated with dorzolamide, and applied, with forceps, to one side of the dorsal surface of the tongue. The control solution (1.26 mM quinine HCl in 1% methyl cellulose) was simultaneously applied, in the same manner, to the opposite side of the tongue; the side receiving dorzolamide or control was counterbalanced across subjects. Because the application of dorzolamide increases salivary flow rate, which might cause confounding effects because of the mixing of chemicals on the lingual surface, a suction device (Saliva Ejector, 6 inch clear; Sullivan Dental Products Inc., Sacramento, CA) was used by subjects to remove excess saliva. Subjects were allowed to use the device at any time, except for the 15 sec immediately before performing the two-alternative forced-choice (2-AFC) or rating tests (see below). This device has been used in previous experiments and was demonstrated to be free of any confounding effects (Dessirier et al., 1997). The filter papers were left on the tongue for 5 min, after which time subjects were asked to rinse their mouth with deionized water. After the rinse procedure, lingual sensitivity to CO_2 was tested by flowing carbonated water at a rate of 20 ml/min bilaterally over the dorsal surface of the tongue for 5 sec on one trial and 15 sec on the next. It was noted in pilot studies that increasing the duration of flow diminished the differences between the treated and untreated sides of the tongue. Because we wanted to maximize sensitivity to the carbonated water stimulus, durations were not counterbalanced across subjects; all subjects completed the 5 sec trial before completing the 15 sec trial.

We used a 2-AFC methodology in which we asked subjects to indicate the side of the tongue perceived to have the strongest sensation elicited by carbonated water during a 5 sec application and the last 5 sec of a 15 sec application. In the previous case, subjects were told to concentrate on the sensation elicited by the carbonated water during the entire 5 sec application, whereas, in the latter case, subjects were told to disregard any sensation during the first 10 sec and compare the sensations elicited on either side of the tongue only during the last 5 sec (at which time they were prompted). In addition to the forced-choice, subjects were also asked, at each time point (5 and 15 sec), to independently rate the intensity of the sensation elicited by carbonated water on each side of the tongue using a 0–10 category scale (0, no sensation; 10, intense sensation).

Although we believed that dorzolamide would selectively attenuate CO_2 irritation by blocking carbonic anhydrase activity, it was conceivable

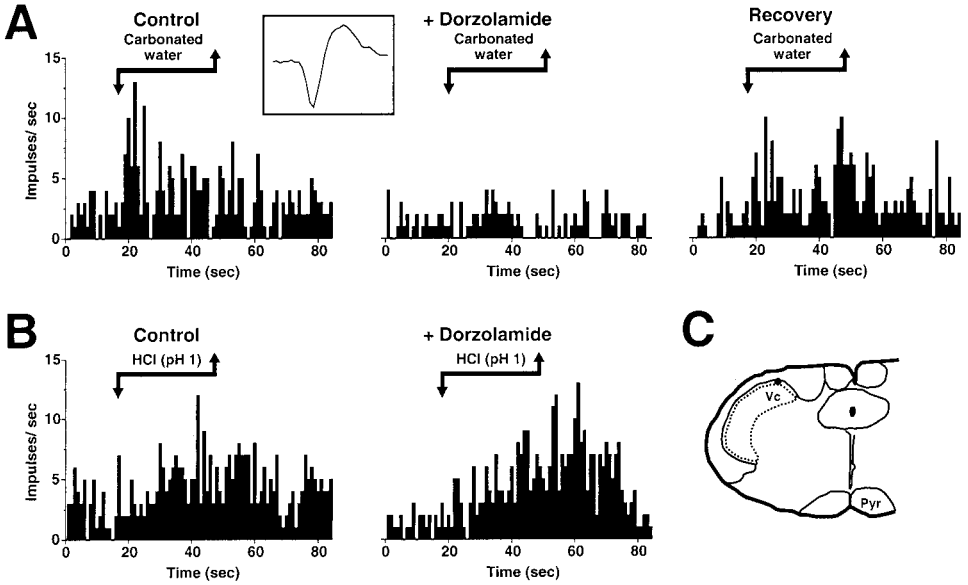

Figure 2. Example of dorzolamide blockade of unit response to carbonated water. *A*, Carbonated water. Left PSTH (bin width, 1 sec) shows response of the unit to carbonated water flowed continuously for 30 sec (*arrows*) on the anterior surface of the tongue. The middle PSTH shows reduction in the response of the same neuron to carbonated water after pretreatment of the tongue with dorzolamide. PSTH on *right* shows recovery of response. *Inset* shows example of action potential waveform. *B*, PSTHs of the responses of the same unit to HCl (*left*) was not reduced after dorzolamide application (*right*). *C*, Recording site (●) on drawing of brainstem section through Vc. Abbreviations as in Fig. 1.

that the treatment could have nonspecific effects, thereby blocking the sensations induced by other chemical or tactile stimuli. Therefore, to test this possibility, separate control experiments were conducted using acid (200 mM pentanoic acid) and tactile stimuli (von Frey filament; 0.229 mN). The pentanoic acid was chosen because it had an oil–water partition coefficient similar to CO_2. The order in which these treatments were given was counterbalanced across subjects. For acidic stimuli, two filter paper disks (1.0 cm diameter; Whatman International Ltd.), each saturated with 15 μl of pentanoic acid, were placed, using forceps, on each side of the tongue in a corresponding area that had been previously treated with dorzolamide or control solution. Subjects were told to close their mouth, and after ~5 sec, the filter papers were removed and subjects were again asked to perform a 2-AFC and indicate the side of the tongue for which a stronger tingling–burning sensation was perceived. They were asked to disregard sour taste when making this judgment. In addition, they again used the same 0–10 category scale to rate the intensity of the sensation perceived on each side of the tongue.

Tactile sensitivity of the tongue was tested by applying the von Frey filament to the dorzolamide-treated side, the untreated (control) side, or not at all (blank), in randomized order. Each condition was tested 10 times for a total of 30 trials. Subjects responded by indicating whether or not they felt the stimulus and whether or not they were sure of their response. From these data, response matrices for each subject were constructed from which indices representing the tactile sensitivity on each side of the tongue were calculated (R-index) (O'Mahony, 1992).

To show that the effect of dorzolamide was present when subjects performed the acid and tactile control tests, carbonated water was applied again at the end of the session. As before, subjects performed a 2-AFC test and rated the intensity of carbonation on the treated and untreated sides of the tongue at 5 sec.

Finally, it is possible that subjects chose a side as having a stronger sensation elicited by carbonation, not because of the perception encountered, but because of some cue elicited by the dorzolamide or control treatment. Thus, at the end of each experimental session, two filter papers (2.5 cm diameter, cut in half) were again saturated with dorzolamide or control solution, placed onto both sides of the tongue in random order, and left for 5–10 sec. After removal of the papers, subjects were asked to select the side they thought contained the chemical responsible for reducing the intensity of the sensation evoked by carbonated water. If no taste or textural cues were consistently used by subjects, it would be expected that subjects would choose randomly between the dorzolamide or control; this would result in each chemical being selected 50% of the time.

Data analysis. A binomial analysis was used to determine whether a significant majority of subjects chose a particular side (dorzolamide-treated vs nontreated) as having a stronger carbonation- or acid-evoked sensation and a d′ analysis (Ennis, 1993) was also performed to determine the strength and significance of the effect using the method of Bi et al. (1997); $p < 0.05$ was considered significant in all cases. To determine whether the intensity of the sensation elicited by carbonated water or pentanoic acid varied significantly between the dorzolamide- and control-treated sides of the tongue, mean intensity scores for each side were calculated under each condition, and a paired Student's *t* test was used. R-indices for the treated and untreated sides of the tongue were calculated, and a *t* test was used to determine whether there was a significant difference between the two.

RESULTS

Experiment 1: electrophysiology

Ten units responded to non-noxious pinch (as assessed by the experimenter when the same stimulus was applied to the webbing between fingers), noxious heat (54°C), and carbonated water and thus were categorized as wide dynamic range. Of these, two also responded to hydrochloric acid, pH 1. Spontaneous activity in these units was generally low, having a discharge frequency that seldom exceeded 5 Hz. The receptive field was usually limited to the tongue, but in three units, also included portions of the ipsilateral upper and/or lower lip and cheek. Recording sites were histologically localized to Vc. A photomicrograph of a lesion site is shown in Figure 1*A*, and all recovered sites are compiled on a representative brainstem section in Figure 1*B*. They were located in the superficial layers of the dorsomedial aspect of Vc ipsilateral to the receptive field, consistent with our previous results (Carstens et al., 1998).

Dorzolamide treatment significantly reduced the response of these units to carbonated water (*t* test; $p < 0.001$). Moreover, the treatment appeared to be specific to acid production from CO_2 because the response from two cells to HCl did not change after dorzolamide application. An example is shown in Figure 2*A* in which the response of a single unit to carbonated water (left PSTH) is reduced after dorzolamide treatment (middle PSTH), followed by recovery of the response (right PSTH). Figure 2*B* shows that the response of this same unit to the acid stimulus (left PSTH) was not affected by pretreatment with dorzolamide (right PSTH).

Averaged PSTHs for the 10 units are shown in Figure 3. No significant differences were seen in the spontaneous firing rate of cells before and after dorzolamide treatment (*t* test; $p = 0.48$). However, the activity induced in cells by carbonated water after dorzolamide was significantly lower compared with the response

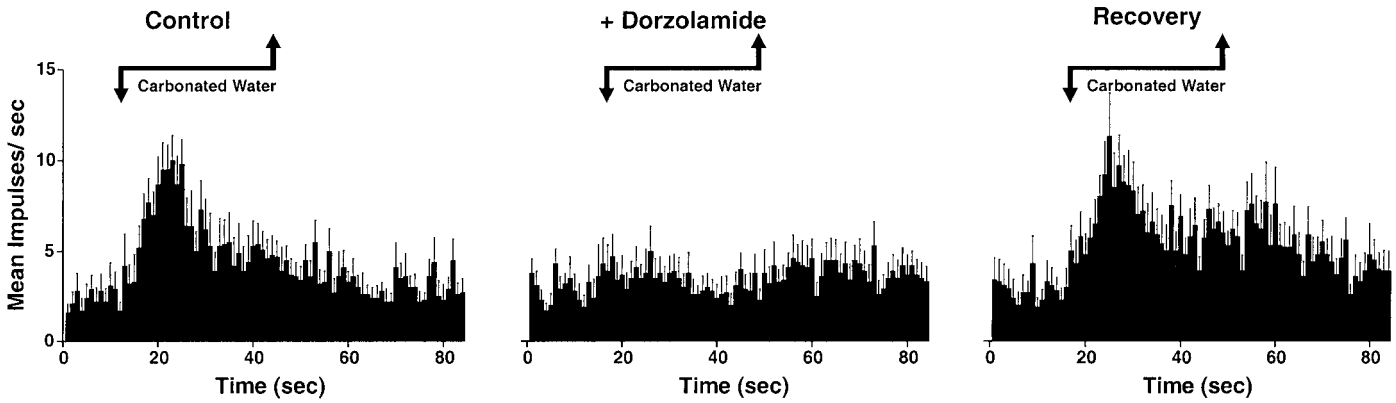

Figure 3. Mean PSTH (bin width, 1 sec) of 10 neurons to carbonated water applied for 30 sec to the dorsal surface of the rat tongue. *Left PSTH*, Mean response before the application of dorzolamide. *Middle PSTH*, Mean response after dorzolamide. *Right PSTH*, Recovery from the effect of dorzolamide. Spontaneous activity was not subtracted. Error bars indicate SEM.

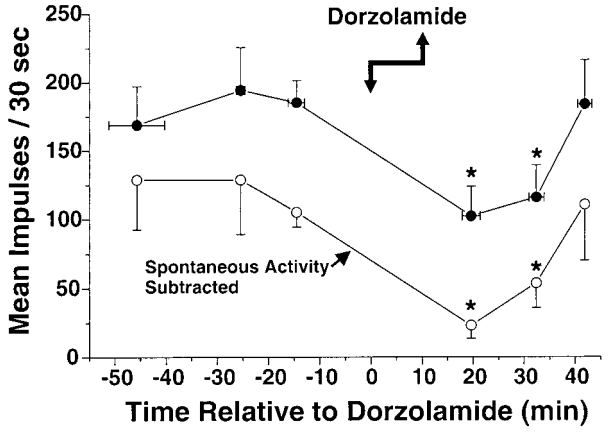

Figure 4. Time course of effect of dorzolamide. Graph plots mean responses of 10 units to application of carbonated water versus time relative to application of dorzolamide (indicated by *arrows*). ●, Spontaneous activity not subtracted; ○, spontaneous activity (total number of impulses during 30 sec before application of carbonated water) subtracted from evoked response (total number of impulses during 30 sec stimulus period). Error bars indicate SEM. $^*p < 0.05$, significantly different from predorzolamide level; *t* test.

of the cells before dorzolamide (*t* test; $p < 0.001$). The effect of dorzolamide treatment was reversible because the response to carbonated water recovered after ~40 min (right PSTH), at which time neither spontaneous activity nor the response to carbonated water were significantly different compared with predorzolamide levels (*t* test; $p = 0.68$ and $p = 0.65$, respectively). Figure 4 plots mean responses to carbonated water versus time, without (●) and with (○) spontaneous activity subtracted from the evoked response. Predorzolamide responses were stable, and responses were significantly reduced at 20 and 30 min after dorzolamide, with recovery at 40 min. When spontaneous activity was subtracted, it is apparent that dorzolamide almost completely abolished the response to carbonated water.

Experiment 2: immunohistochemistry

Representative photomicrographs of FLI in the regions analyzed are shown in Figure 5; *boxes* in the drawings of brainstem sections indicate each corresponding region. Application of carbonated water elicited significantly higher FLI in cells located in the dorsomedial aspect of Vc compared with the application of saline (*t* test; $p < 0.05$), flat water (*t* test; $p < 0.05$), or no stimulation (*t* test; $p < 0.05$). Figure 6*A* shows a photomicrograph of cell nuclei expressing FLI in this region after the application of carbonated water. The brainstem distribution of FLI in one rat subjected to carbonated water treatment is shown in Figure 7*A*. FLI was concentrated in the dorsomedial Vc bilaterally, as well as in ventrolateral Vc, the ventrolateral medulla, and throughout the rostrocaudal extent of NTS bilaterally.

Pretreatment with dorzolamide resulted in a significant reduction in FLI in the dorsomedial Vc. The photomicrograph in Figure 6*B* of dorsomedial Vc from a dorzolamide-pretreated rat shows a marked reduction in FLI compared with application of carbonated water alone (Fig. 6*A*). Figure 7*B* shows the brainstem distribution of FLI from a dorzolamide-pretreated rat. Note in particular the marked reduction in FLI in the dorsomedial Vc throughout its rostrocaudal extent. This was borne out in the mean FLI counts. Figure 8*A* plots mean counts of FLI in dorsomedial Vc for each treatment group and shows that the dorzolamide pretreatment, as well as the saline, flat water, and unstimulated control groups, all showed significantly less FLI compared with the carbonated water group. There was also significantly more FLI in the ventrolateral aspect of Vc in the carbonated water group compared with saline or unstimulated controls (Fig. 8*B*). There was a trend toward lower FLI in the dorzolamide group, although this failed to reach statistical significance (Fig. 8*B*). There were no other between-group differences in FLI in any of the other regions analyzed, except in NTS in which the unstimulated control group showed significantly less FLI compared with the other groups (Fig. 8*D*).

To control for the possibility that the stimulus application procedure elicited FLI, saline and flat water flow controls were undertaken. The photomicrographs in Figure 6 show that application of saline (Fig. 6*C*) and flat water (Fig. 6*D*) resulted in some FLI in dorsomedial Vc. Figure 7*C* shows the brainstem distributions of FLI in an individual rat subjected to flat water. The distribution of FLI was qualitatively similar to that evoked by carbonated water (Fig. 7*A*), except that there was significantly less FLI in dorsomedial Vc (Fig. 8*A*). In addition, an unstimulated control group was included to separately assess possible effects of anesthesia and passage of time. There was a total absence of FLI in dorsomedial Vc (Fig. 8*A*), and significantly lower FLI in the ventrolateral caudalis (Fig. 8*B*) and NTS (Fig. 8*D*) in this group compared with rats receiving carbonated water. These control

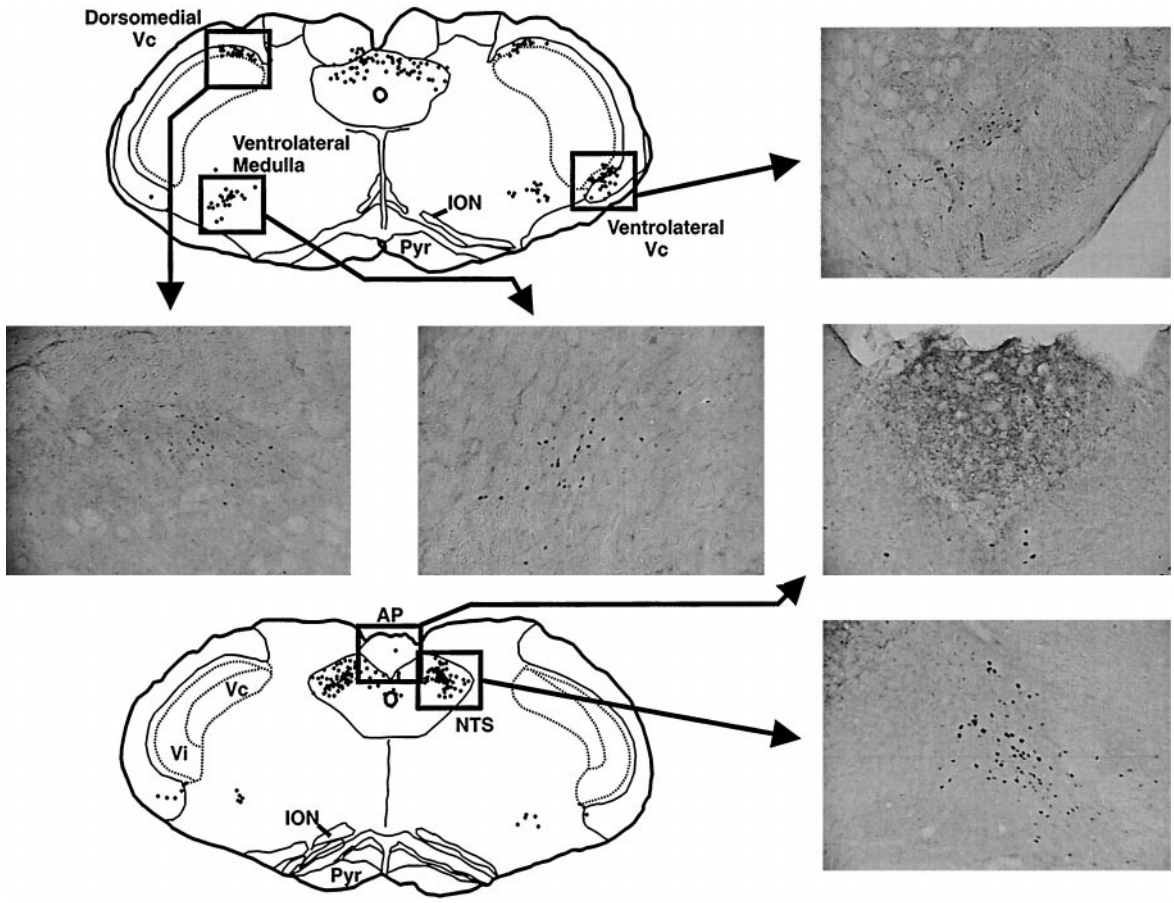

Figure 5. Photomicrographs of regions analyzed for FLI. Each *box* in *top* and *bottom* drawings of brainstem sections corresponds to photomicrograph (40×) showing distribution of FLI (*black*) within that region. Abbreviations as in Figure 7.

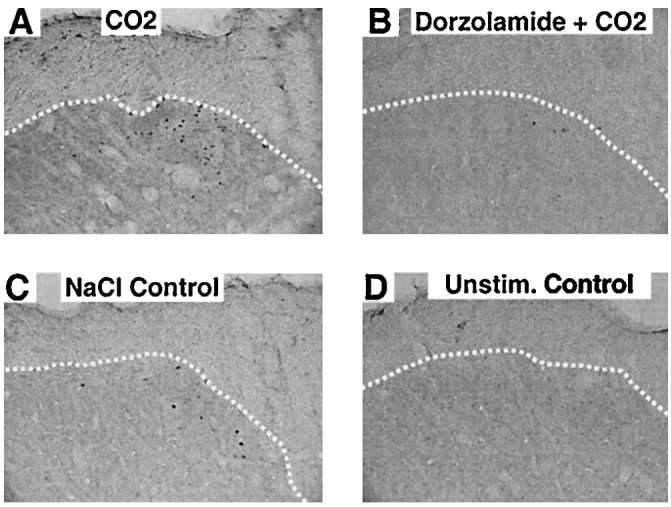

Figure 6. Photomicrographs showing distribution of FLI in the dorsomedial aspect of Vc. *A*, Carbonated water only. *B*, Pretreatment with dorzolamide, followed by carbonated water. *C*, Isotonic saline (0.9%) applied to tongue in same manner as dorzolamide. *D*, Flat water flowed in the same manner as carbonated water.

data indicate that the stimulus application procedures probably induced some FLI by mechanical stimulation of the tongue and/or gustatory effects in NTS, but that this degree of FLI was nonetheless significantly less than that evoked by carbonated water.

Experiment 3: psychophysics

Carbonated water, when flowed over the tongue for 5 sec, elicited a stronger sensation on the side of the tongue not treated with dorzolamide in a significant majority of subjects (18 of 21; binomial; $p < 0.001$; equivalent to a d′ value of 1.51; $p < 0.01$) (Fig. 9*A*). When asked to rate the intensity of the carbonation sensation, significantly lower ratings were assigned to the dorzolamide-treated side compared with the untreated side (4.7 ± 0.3 vs 6.2 ± 0.3; *t* test; $p < 0.001$). However, when carbonated water was flowed for 15 sec, the number of subjects selecting the nontreated side as having the stronger sensation was reduced and no longer significant (14 of 21; binomial; $p = 0.19$) (Fig. 9*B*). The equivalent d′ value of 0.61 was not significant ($p = 0.13$). Moreover, after the longer flow, the intensity rating of the treated side reached a level equal to that seen on the untreated side (6.2 ± 0.3 vs 6.2 ± 0.4, respectively; *t* test; $p = 1.0$).

When the same experiment was conducted using pentanoic acid, the intensity of sensation on the treated and untreated sides of the tongue was similar, indicating that the dorzolamide treatment selectively attenuated the irritation induced by CO_2 but not acid (Fig. 9*C*). Thus, the proportion of subjects choosing the nontreated side as yielding a stronger irritation was not a significant majority (11 of 21; binomial; $p = 0.66$), and the d′ value (0.085) was not significant ($p = 0.82$). In addition, the mean intensity rating elicited by the acid on the dorzolamide-treated side was not significantly different from that on the untreated side (4.2 ± 0.4 vs 4.2 ± 0.4; *t* test; $p = 1.0$).

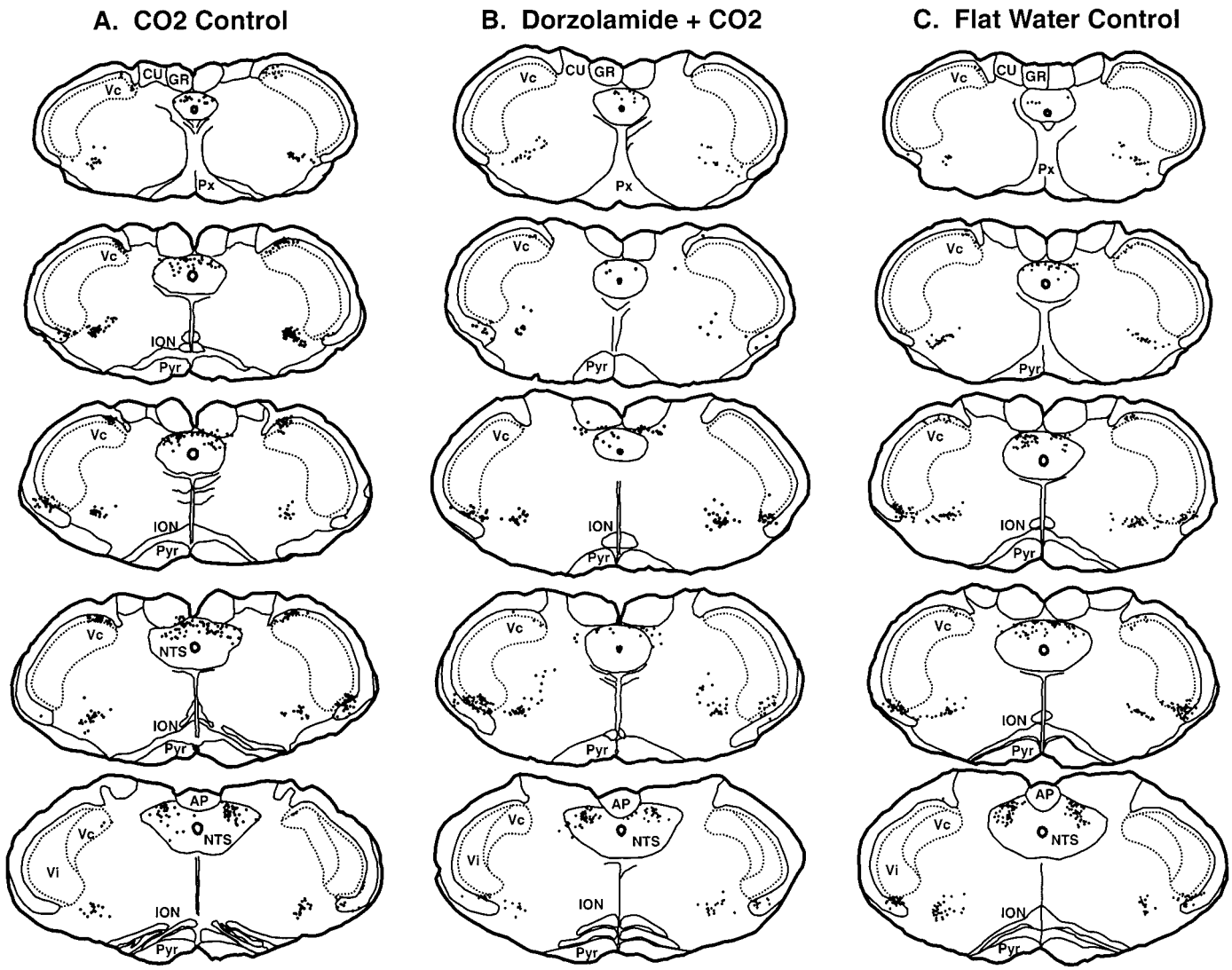

Figure 7. Brainstem distribution of FLI. *A*, Brainstem sections from a rat that received carbonated water only, arranged from caudal (*top*) to rostral (*bottom*). *Black dots* indicate cell nuclei exhibiting FLI. Lateral and medial *dashed lines* indicate approximate borders of laminae I–II and ventral Vc, respectively. *B*, Brainstem sections as in *A* from rat receiving dorzolamide, followed by carbonated water. *C*, Sections from rat receiving flat (uncarbonated) water as a control. *CU*, Cuneate nucleus; *GR*, nucleus gracilis; *ION*, inferior olivary nucleus; *Pyr*, pyramid; *Vi*, trigeminal nucleus interpolaris.

Pretreatment with dorzolamide had no measurable effect on the tactile sensation produced by the von Frey hair applied to the dorsal surface of the tongue. Mean R-indices representing the tactile sensitivity on the treated and nontreated side were not significantly different (72 vs 71%, respectively; *t* test; $p = 0.44$). These measures confirmed that there was no difference in tactile sensitivity between the two sides of the tongue.

After the tasks involving pentanoic acid and tactile sensitivity, we retested the subjects with carbonated water to determine whether the action of the dorzolamide was maintained over the entire test session (30 min). When carbonated water was delivered for 5 sec, a significant majority of subjects again selected the untreated side as having the stronger sensation (16 of 21; binomial; $p < 0.03$); this is equivalent to a significant d′ value of 1.01 ($p < 0.02$). Similarly, subjects assigned significantly higher intensity ratings to the untreated side (5.5 ± 0.4) compared with the dorzolamide-treated side (4.7 ± 0.3; *t* test; $p < 0.05$).

Finally, to control for the effects of taste or texture bias on the collected results, we asked subjects to report which chemical they thought was responsible for reducing the intensity of the sensation evoked by carbonated water. Forty-eight percent (10 of 21) of the subjects selected dorzolamide and 52% (11 of 21) selected the control solution, thus ruling out a bias in texture or taste as influencing the psychophysical results obtained in this study.

DISCUSSION

This study investigated the origin of the sensation elicited by the oral application of carbonated water using three complementary methodologies: electrophysiological recordings of single units in Vc, c-Fos immunohistochemistry, and human psychophysics. In each case, the neural activity or perception induced by carbonated water was attenuated by previous treatment of the tongue with the carbonic anhydrase inhibitor dorzolamide. These results independently confirm the chemogenic nature of irritation produced by carbonated water on the tongue.

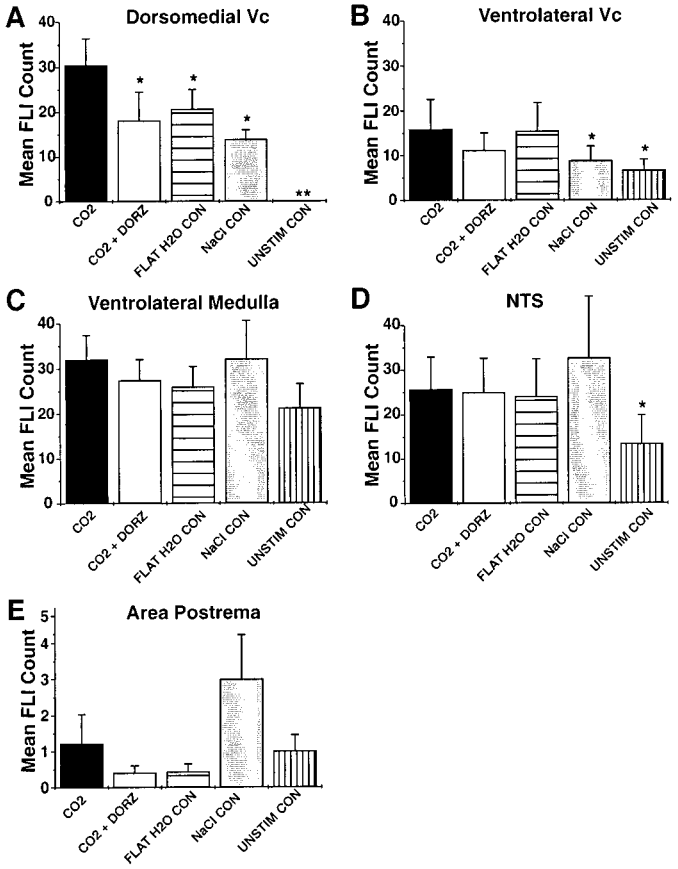

Figure 8. Mean counts of cell nuclei expressing FLI for each region analyzed. *A*, Bar graph plots mean number of cell nuclei expressing FLI in dorsomedial Vc for each treatment group. CO_2, carbonated water only; CO_2 + *DORZ*, pretreatment with dorzolamide, followed by carbonated water; *FLAT H_2O CON*, flat (uncarbonated) water applied in same manner as carbonated water; *NaCl CON*, 0.9% saline applied in same manner as dorzolamide; *UNSTIM CON*, unstimulated control. $n = 5$–11 rats per group. Error bars indicate SEM. $^*p < 0.05$, significantly different from CO_2 group; unpaired *t* test. *B–E*, Bar graphs as in *A* for indicated brainstem regions of interest.

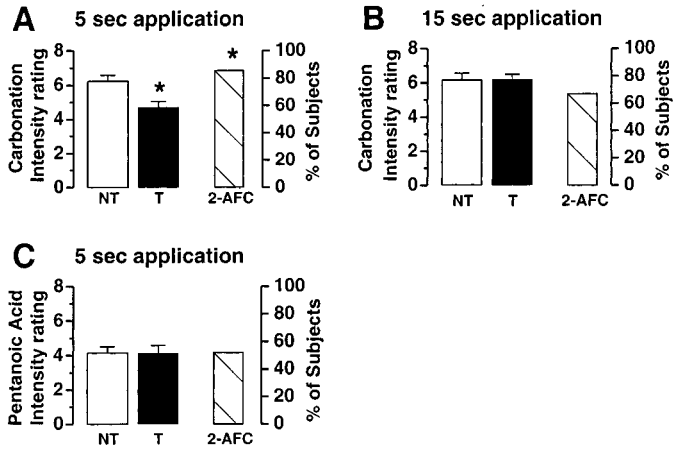

Figure 9. Psychophysical results. *A*, Bar graph to the *left* gives intensity ratings of carbonation for the nontreated (*NT*, *open bar*) and dorzolamide pretreated (*T*, *filled bar*) side of the tongue, respectively. The *hatched bar* to the *right* indicates the proportion of subjects who chose the nontreated side to yield a stronger sensation in the 2-AFC test. Ratings were made 5 sec after application of carbonated water to the tongue by continuous flow. Error bars indicate SEM. $^*p < 0.05$ (*filled bars*), significant difference between pretreated and nontreated; *t* test. $^*p < 0.05$ (*hatched bars*), significant majority of subjects chose nontreated side; binomial test. *B*, Graphs as in *A* for judgements made 15 sec after application of carbonated water. *C*, Graphs as in *A* showing lack of effect of dorzolamide pretreatment on irritation evoked by pentanoic acid when applied for 5 sec to the tongue.

Activation of Vc neurons by carbonated water

The trigeminal nuclear complex is a major relay in the processing of orofacial nociceptive information. Although Vc has been most extensively studied (for review, see Sessle and Greenwood, 1976; Hu et al., 1981; Dubner and Bennett, 1983), the more rostral subnuclei interpolaris and oralis are also involved (Greenwood and Sessle, 1976; Sessle and Greenwood, 1976; Nord and Young, 1979; Hu et al., 1981, 1992; Hayashi et al., 1984; Hu and Sessle, 1984; Hayashi and Tabata, 1989; Dallel et al., 1990, 1996; Jacquin and Rhoades, 1990; Raboisson et al., 1991; Ohya, 1992; Ohya et al., 1993). Vc processes information from mechanosensitive, proprioceptive, thermosensitive, and nociceptive fibers originating in the orofacial region (Hu et al., 1981; Sessle et al., 1981; Bushnell et al., 1984; Hu, 1990; Chiang et al., 1994; McHaffie et al., 1994; Raboisson et al., 1995), including the oral cavity (Kruger and Michel, 1962; Yokota, 1975; Amano et al., 1986; Strassman and Voss, 1993; Carstens et al., 1995, 1998), nasal sinus (Cain and Murphy, 1980; Garcia Medina and Cain, 1982; Cometto-Muñiz and Noriega, 1985; Stevens and Cain, 1986; Anton et al., 1991a,b, 1992; Thürauf et al., 1991, 1993; Shusterman and Balmes, 1997a,b), and cornea (Belmonte and Giraldez, 1981; Belmonte et al., 1991; Pozo and Cervero, 1993; Bereiter et al., 1994; Chen et al., 1995, 1997; Bereiter and Bereiter, 1996; Meng and Bereiter, 1996; Meng et al., 1997; Carstens et al., 1998). We presently found that wide dynamic range-type Vc units additionally responded to lingual application of carbonated water, indicating that this stimulus is capable of activating trigeminal nociceptive pathways. Furthermore, Vc responses were significantly attenuated by local pretreatment with the carbonic anhydrase inhibitor dorzolamide in a manner that was selective for CO_2 but not other acids. These data confirm previous reports that CO_2-sensitive primary afferents in the lingual nerve (Komai and Bryant, 1993) and the chorda tympani (Komai et al., 1994) are inhibited by previous application of carbonic anhydrase inhibitors and extend them by showing a selective, carbonic anhydrase-dependent excitatory action of carbonated water at the level of Vc neurons. Thus, the conversion of CO_2 to carbonic acid appears to be a requisite step for the excitation of primary nociceptive afferents that transmit signals on CO_2 irritation to Vc and higher centers.

Possible mechanisms underlying actions of CO_2 and acids

Not all Vc units that responded to carbonated water also responded to HCl. Similar results have also been reported for lingual nerve fibers (Komai and Bryant, 1993). One possible explanation is that CO_2, being a small lipophilic molecule, can readily diffuse through the lingual epithelium and membranes of nociceptive fiber terminals. Thus, CO_2 might be capable of activating nociceptors located in deeper layers of the tongue, which are less accessible by protons generated from acids such as HCl that dissociate at the lingual surface. However, many organic acids are lipophilic, and Bryant and Moore (1995) reported that the efficacy of fatty acids to excite lingual nerve fibers increased as a function of lipophilicity (i.e., carbon chain length). We wished to determine whether more lipophilic acids, comparable with

CO_2 in their ability to penetrate the lingual epithelium, act in a carbonic anhydrase-independent manner. One of these, pentanoic acid, elicited an irritant sensation that was unaffected by dorzolamide, in marked contrast to carbonated water (Fig. 9). This indicates that carbonated water and acidic stimuli activate lingual nociceptors via distinct carbonic anhydrase-dependent and -independent mechanisms, respectively.

It is currently not known whether the carbonic acid formed from CO_2 acts intracellulary or extracellularly to excite lingual nerve fiber terminals. In the former instance, CO_2 would diffuse through the membrane of nociceptor terminals to encounter carbonic anhydrase, which would effect an intracellular acidification resulting in neuronal activation. The presence of carbonic anhydrase within trigeminal ganglion neurons (Wong et al., 1983; Neubauer, 1991) lends support to this hypothesis. Hydrophilic acids such as HCl would be unable to pass through the cell membrane, requiring instead some extracellular mechanism to excite nociceptors (see below). There is little direct support for the idea of intracellular acidification. In taste receptor cells, increased extracellular pCO_2 (with extracellular pH held constant) produced a transient reduction in intracellular pH (Lyall et al., 1997), but it is not known whether this depends on intracellular carbonic anhydrase or whether a similar mechanism exists in nociceptors. Alternatively, CO_2 may be converted into carbonic acid extracellularly. Carbonic anhydrase is present in saliva (Feldstein and Silverman, 1984; Murakami and Sly, 1987; Fernley et al., 1991). The recent identification of acid-sensing ion channels (ASIC) in sensory neurons could provide the link between extracellular acidification and pain associated with CO_2 application (Waldmann et al., 1997b). Extracellular conversion of CO_2 into carbonic acid would cause a localized increase in concentration of protons, which could then activate nociceptors via gating of ASIC or dorsal root acid-sensing ion channels presumably located in the membrane of nociceptor fiber terminals (Lingueglia et al., 1997; Waldmann et al., 1997a). Additional transduction mechanisms, such as direct depolarization of nociceptor terminals via proton influx through proton or Na^+ channels, may also participate in the activation of nociceptors by acidic stimuli.

Immunohistochemistry

The c-Fos immunohistochemical studies confirmed the electrophysiological results by showing that the FLI induced by carbonated water in dorsomedial Vc is significantly reduced by pretreating the tongue with dorzolamide. This provides further support for the chemogenic nature of sensations elicited by carbonated water.

The Vc shares attributes with the dorsal horn of the spinal cord (Dubner and Bennett, 1983). Superficial layers of Vc are analogous to laminae I–II in the dorsal horn, whereas the magnocellular region corresponds to spinal dorsal horn layers III–IV (Gobel et al., 1988). Nociceptive neurons are found in the superficial layers of Vc (Pozo and Cervero, 1993; Meng et al., 1997; Carstens et al., 1998). Our data support the critical role for Vc in mediating oral CO_2 irritation because it, but not other brainstem regions, showed a significant decrease in FLI after dorzolamide application.

Counts of FLI in the NTS were significantly lower in the unstimulated control group compared with all others (Fig. 8*D*), suggesting that mechanical (e.g., flow), as well as chemical irritant (CO_2) or taste (NaCl) aspects of the stimuli, may have contributed to FLI in NTS.

Psychophysics

The present psychophysical data corroborate the neurobiological results by showing that the sensation elicited by carbonated water is significantly attenuated by pretreatment with dorzolamide. That dorzolamide treatment did not affect irritation induced by pentanoic acid, or tactile sensitivity, indicates that its effect was selective for carbonated water and was not attributable to a nonspecific anesthetic action. Thus, these data support the emerging hypothesis that the perception of oral carbonation is attributable to the conversion of CO_2 into carbonic acid, which is then capable of exciting lingual chemosensitive nociceptors projecting to Vc (Green, 1992; Komai and Bryant, 1993; Komai et al., 1994; Carstens et al., 1998).

When the carbonated water was flowed for 15 sec compared with 5 sec, subjects no longer perceived the dorzolamide-pretreated side of the tongue as having a weaker sensation (Fig. 9*B*). This was not caused by a washout of dorzolamide, because subjects thoroughly rinsed before the initial 5 sec test with carbonation yet still reported significant differences between the two sides of the tongue. Furthermore, when the carbonation test was repeated (after intervening tests for acid and tactile sensation), a significant majority still chose the nontreated side as having the stronger sensation. One possible explanation is that dorzolamide, although lipophilic, is not as membrane-permeable as CO_2. Therefore, with a longer stimulus period, CO_2 might activate more distant nociceptors that dorzolamide did not reach. Also, as the duration of CO_2 stimulation increases, other factors, such as stimulation of mechanoreceptors, may contribute to the perception of carbonation. In support of this, subjects never assigned an intensity rating of zero to the dorzolamide-treated side, although this might also be explained by incomplete inhibition of carbonic anhydrase or subject response bias. Finally, subjects rated the intensity of the untreated side to be equal in magnitude for both the 5 and 15 sec stimulation periods. This runs counter to what would be expected because, as CO_2 penetrates deeper into epithelial tissue, it should recruit more nociceptors, evoking a stronger sensation because of spatial summation. Speculatively, however, nociceptive fibers might desensitize to CO_2 at a rate equal to which new fibers are recruited.

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