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Sensory Properties of Citric Acid: Psychophysical Evidence for Sensitization, Self-desensitization, Cross-desensitization and Cross-stimulus-induced Recovery Following Capsaicin

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Abstract

In a first experiment, human subjects used a bipolar scale to rate the irritant sensation elicited by 10 sequentially repeated applications of either 3 ppm capsaicin or 250 mM citric acid on one side of the dorsal surface of the tongue, at 1 min intervals (30 s inter-stimulus interval). Citric acid-evoked irritation significantly increased across trials, consistent with sensitization. With capsaicin there was a large degree of inter- and intra-individual variation in successive ratings with no overall sensitization. Following the sequential stimulation series and a 10 min rest period, self- and cross-desensitization effects were tested in a two-alternative forced choice (2-AFC) paradigm by placing either citric acid or capsaicin on both sides of the tongue and asking subjects to indicate which side of the tongue yielded a stronger irritant sensation. Subjects also gave separate intensity ratings for irritation on each side of the tongue. Capsaicin self-desensitization was confirmed, while cross-desensitization to citric acid was not observed. In addition, citric acid self-desensitization and cross-desensitization to capsaicin were observed. In a second experiment a stronger capsaicin solution (33 ppm) was applied to one side of the tongue using cotton swabs. After the burning sensation elicited by capsaicin had disappeared, citric acid was applied bilaterally and cross-desensitization was observed using the same 2-AFC and rating procedures. This was followed by repeated re-application of citric acid at 1 min intervals to the capsaicin-treated side. The irritant sensation elicited by citric acid increased significantly, indicating a 'cross-stimulus-induced recovery' from capsaicin desensitization. In a final experiment we investigated the effect of the sodium channel blocker amiloride on the perceived irritation elicited by citric acid or capsaicin. Following application of amiloride to one side of the tongue with cotton swabs, either citric acid or capsaicin was applied bilaterally and subjects asked to perform a 2-AFC and intensity ratings. Amiloride significantly, albeit weakly, reduced the irritation elicited by citric acid while it weakly but significantly enhanced capsaicin-evoked irritation. These findings are discussed in terms of involvement of vanilloid and acid-sensitive ion channels in acid-evoked irritation and pain.

Introduction

Capsaicin, the pungent principle in red chili pepper, induces irritation (burning, stinging/pricking sensations) as well as pain when applied to mucosae and skin (Green, 1990; Szolcsànyi, 1990; Geppetti *et al.*, 1993). Such sensations are thought to be governed by the action of capsaicin on a 'capsaicin' vanilloid receptor, a subtype of which was sequenced and named VR1 (Caterina *et al.*, 1997). While possessing a binding site for capsaicin, VR1 also appears to detect the presence of noxious heat as well as acidic conditions (Tominaga *et al.*, 1998).

Acids, in addition to stimulating the gustatory system to elicit sour tastes, also stimulate the chemesthetic sense, inducing sensations of irritation and pain when applied to the oral (Gilmore and Green, 1993), nasal (Anton *et al.*, 1992; Geppetti *et al.*, 1993; Cometto-Muñiz *et al.*, 1998) and ocular (Chen *et al.*, 1995) mucosae as well as skin (Steen and Reeh, 1993; Green and Bluth, 1995; Steen *et al.*, 1995a). Neurophysiologically, acidic stimuli excite lingual nerve (Komai and Bryant, 1993; Bryant and Moore, 1995) and corneal (Belmonte *et al.*, 1991; Pozo *et al.*, 1992; Gallar *et al.*, 1993; Chen *et al.*, 1997) afferent fibers, as well as peripheral cutaneous nociceptors (Steen *et al.*, 1992, 1995b) and central nociceptive neurons in the trigeminal complex (Peppel and Anton, 1993; Strassman and Vos, 1993; Martinez and Belmonte, 1996; Carstens *et al.*, 1998).

Many observations support the involvement of a vanilloid receptor in acid-induced pain and irritation [for a review see (Bevan and Geppetti, 1994)]. For instance, acidic stimulation of the upper airway in guinea pigs induces coughing and other behavioral reactions that are reduced by the capsaicin antagonist capsazepine (Lou and Lundberg, 1992; Lalloo *et al.*, 1995). In addition, acids are thought to

excite nociceptive endings by binding to proton-gated ion channels that are members of the amiloride-sensitive Na^+ channel/degenerin family (ASIC, for acid-sensitive ion channels), several subtypes of which were recently identified (Bassilana *et al.*, 1997; Lingueglia *et al.*, 1997; Waldmann *et al.*, 1997a,b; Chen *et al.*, 1998) [for the latest nomenclature see (Waldmann and Lazdunski, 1998; Waldmann *et al.*, 1999)].

Capsaicin is known for its ability to induce desensitization (Jansco *et al.*, 1961; Szolcsànyi, 1990). Thus, after an initial capsaicin stimulus applied to the tongue, followed by a 5–15 min waiting period, the irritant sensation induced by subsequent application of capsaicin or other irritant chemicals is reduced (self- and cross-desensitization, respectively) (Green, 1989, 1991a, 1993; Gilmore and Green, 1993; Cliff and Green, 1996; Dessirier *et al.*, 1997). The cellular mechanisms underlying capsaicin self-desensitization have not yet been fully delineated, but appear to require influx of Ca^{2+} to possibly activate intracellular proteases (Cholewinski *et al.*, 1993; Chard *et al.*, 1995). Cross-desensitization represents a more general Ca^{2+}-dependent alteration of nociceptor function (Bevan and Szolcsányi, 1990) to possibly result in depletion of neurotransmitter (Buck and Burks, 1986; Holzer, 1991), interference with repletion by blockade of NGF intra-axonal transport [for a recent review see (Szallasi and Blumberg, 1999)], blockade of voltage-sensitive Na^+ channels (Su *et al.*, 1999) or even C fiber degeneration (Simone *et al.*, 1998). Most irritant chemicals tested to date exhibit self-desensitization (Cliff and Green, 1994; Prescott and Stevenson, 1996a,b; Dessirier *et al.*, 1997, 1999; Prescott, 1998). Piperine, a vanilloid compound which shares similar properties with capsaicin (Martenson *et al.*, 1994, 1997; Liu and Simon, 1996), also exhibits cross-desensitization to capsaicin (Green, 1996; Dessirier *et al.*, 1999). However, cross-desensitization to capsaicin or other irritants has not been shown for the few other irritant chemicals tested to date: zingerone (Szolcsányi and Jancsó-Gábor, 1976; Gilmore and Green, 1993), nicotine (Dessirier *et al.*, 1997, 1999) and menthol (Cliff and Green, 1996). Conceivably, higher concentrations of these irritants are required to induce cross- as compared with self-desensitization, as suggested by recent evidence that a higher nicotine concentration (300–600 mM) can induce cross-desensitization of spinal neuronal responses to histamine (Jinks and Carstens, 1999) or irritant sensations elicited by capsaicin on the tongue (Dessirier *et al.*, 2000b). Such studies suggest that high doses of irritants like nicotine might induce sufficiently large alterations in nociceptor responsiveness to result in cross-desensitization.

Irritant chemicals have also been classified according to their capacity to induce sensitization and stimulus-induced recovery (SIR). Sensitization is an increase in irritant sensation across repeated trials of application at short intervals (Green, 1989, 1991b, 1993; Dessirier *et al.*, 1997, 1999). SIR is a similar increase in irritant sensation across trials of repeated application of the chemical on the lingual epithelium or skin that had been previously desensitized by that same irritant chemical (Green, 1996; Green and Rentmeister-Bryant, 1998). Interestingly, compounds that induce sensitization and SIR in a majority of subjects, such as capsaicin and piperine (Green, 1996; Dessirier *et al.*, 1999), also induce cross-desensitization. In contrast, other irritants such as zingerone, a vanilloid that also has effects similar to capsaicin on trigeminal ganglion cells (Liu and Simon, 1996), primarily induce a progressive decline in irritant intensity upon repeated stimulation (self-desensitization) (Prescott and Stevenson, 1996a,b) but not cross-desensitization (Gilmore and Green, 1993; Green, 1993, 1996). The same appears to be true for lower concentrations of nicotine and for menthol (Cliff and Green, 1994, 1996; Dessirier *et al.*, 1997). The different sensory properties of these irritants, compared with those exhibiting sensitization and SIR (i.e. capsaicin and piperine), might thus reflect different kinetic properties at the molecular receptor level.

This first set of experiments investigated the sensory properties of citric acid on the tongue, specifically sensitization, SIR and self- and cross-desensitization effects. We hypothesized that if acid-induced irritation is mediated via vanilloid receptors such as VR1, it should elicit sensitization and SIR similar to capsaicin. Furthermore, irritation elicited by citric acid would be predicted to exhibit cross-SIR to capsaicin, a possibility tested in the second experiment. Alternatively, citric acid-induced irritation might involve ASICs and thus would be predicted to be amiloride-sensitive. In a third experiment, we thus tested if irritation elicited by citric acid, but not capsaicin, was reduced by amiloride.

Experiment I

Materials and methods

Subjects

Twenty-six healthy individuals (seven males, 19 females, aged 18–27 years), students and staff at the University of California at Davis, volunteered to participate in the study. All refrained from eating or drinking for at least 1 h prior to each experimental session. In addition, subjects were asked not to eat spicy food for 2 days prior to testing. To verify this, subjects were asked to answer questions regarding their food intake over the previous 48 h.

Chemical stimuli and application procedure

All chemicals were purchased from Sigma Chemical Co. (St Louis, MO) unless otherwise specified. A 0.1% (w/v) capsaicin stock solution (98–100% pure) was made up in 95% ethanol and a 3 ppm (10 μM) capsaicin solution was made by diluting the stock solution with dH_2O. Fifteen microliters of this solution was pipetted onto small (78.5 mm^2) and 40 μl onto large (176.7 mm^2) filter paper disks (Whatmann, Maidstone, UK). To avoid any

stimulating effect of ethanol, the filter papers were air dried and then soaked with the same volumes of distilled water immediately prior to application. Citric acid (reagent grade; Baker Chemical Co., Phillipsburg, NJ) was dissolved in distilled water to a concentration of 250 mM and was pipetted in the same volumes onto the small or large filter papers just prior to application.

Throughout the experiment a suction device (Saliva Ejector, 6" clear; Sullivan Dental Products, Sacramento, CA) was placed in the mouth to remove saliva. This freed the subject from having to swallow or spit and avoided spreading the chemical solution across the tongue. Subjects were instructed to use the suction device at any time, except for the 15 s period prior to giving intensity ratings or two-alternative forced choice (2-AFC) responses. This avoided a possible influence of cooling caused by opening the mouth.

Experimental design

Control of matching intensities evoked by capsaicin and citric acid. All subjects participated in an initial session to test whether the concentration selected for capsaicin yielded an intensity of irritation that was approximately equivalent to that of citric acid. It was necessary to check for an approximate match in intensity in a separate session because the intensity rating scale used in later sessions was 'relative' (all intensity ratings always started at 1; see below) and thus would not provide this information. To test that the concentrations were approximately matched in intensity, two large filter paper disks, one soaked with 3 ppm capsaicin and the other with 250 mM citric acid, were placed on corresponding sites on each side of the dorsal anterior surface of the tongue. The sides receiving capsaicin or citric acid were counterbalanced across subjects. After 15 s, subjects performed a 2-AFC test (Green and Swets, 1966) in which they were asked to report which side of the tongue yielded a stronger sensation. Subjects then rated each side separately for irritation intensity using a category scale with two labels, 0 (no sensation) at one end and 10 (intense irritation) at the other end. The 15 s wait was chosen (rather than 30 s as in previous studies; Dessirier *et al.*, 1999) because pilot tests had demonstrated that citric acid-evoked irritation increased very rapidly but was short lived. This was thus an attempt to monitor the peak irritant sensation induced by the citric acid solution. In addition, to assess dynamic changes in intensity over time, 2-AFC and rating tasks were repeated at 30 s intervals for a total period of 3 min. The filter papers were left on the tongue for the entire 3 min period.

Sequential stimulation and desensitization. After the initial session to verify that the intensities of capsaicin and citric acid were approximately matched, four additional experimental sessions followed, each separated by at least 2 days. Each session consisted of two parts: firstly, an initial repeated stimulation on one side of the tongue and, secondly, bilateral stimulation with a 2-AFC test. In the first part, the larger size filter paper disk containing 40 µl of either 3 ppm capsaicin or 250 mM citric acid was applied with forceps to one side of the anterior dorsal surface of the tongue. Thirty seconds after stimulus onset, the filter paper was removed. After a further 30 s a fresh filter paper containing the same stimulus (capsaicin or citric acid) was applied to the same area of the tongue, then removed 30 s later; the inter-stimulus interval (ISI) from offset of the first stimulus to onset of the next was thus 30 s. This was repeated 10 times. Thus, a new filter paper was applied for 30 s at successive 1 min intervals for a total of 10 repetitions. For each of the 10 filter paper applications, subjects were asked to provide a rating of the perceived oral irritation 15 s after stimulus onset when citric acid was the stimulus and 25 s after stimulus onset when capsaicin was the stimulus. The reason for this difference was to collect ratings at the peak of the sensation for each stimulus compound. Ratings were made using a bipolar category scale as described in a previous study (Dessirier *et al.*, 1999) (see below).

After the 10 unilateral sequential stimulus applications, the subjects rested for 10 min. During the waiting period the subjects sat quietly without speaking. Then two small sized filter papers containing either the same chemical as applied previously (self-desensitization) or the other chemical (cross-desensitization) were applied simultaneously with two forceps to the treated side and the corresponding site on the contralateral side. Subjects then performed a 2-AFC test and intensity rating (see below).

Thus, after the matching session subjects participated in four further sessions consisting of two sessions with unilateral sequential application of capsaicin followed by bilateral capsaicin or citric acid stimulation ('cap–cap' or 'cap–cit' sessions, respectively) and two sessions with unilateral sequential citric acid stimulation followed by bilateral application of citric acid or capsaicin ('cit–cit' or 'cit–cap' sessions, respectively).

Rating procedures

Bipolar scale. When stimuli were repeatedly applied to one side of the tongue, subjects rated the intensity of the chemically evoked sensation using a bipolar category scale that was designed specifically to study changes in perceived intensity of sensation during successive stimuli (Dessirier *et al.*, 1999). The protocol used here was very similar to that used in our previous study (Dessirier *et al.*, 1999) and is thus only briefly described. The bipolar scale consists of a set of category scales, all printed on a single sheet of paper, with a category labeled 1 in the center and categories ranging from 2 to 15 to the right and from 2 to 15 to the left (Dessirier *et al.*, 1999). The intensity of the initial sensation elicited by the first stimulus was automatically designated as a category rating of 1 in the center of the first scale. The sensation elicited by the second stimulus was made on the second scale by assigning a higher number to the right if the sensation

was stronger or a higher number to the left if the sensation was weaker, compared with the first. Subsequent ratings were made in this manner for each of 10 filter paper applications. Subjects had access to their previous intensity ratings during the session, an approach that has been shown to reduce errors of estimation (Kim and O'Mahony, 1998). Possible advantages of this scale for sequential ratings have been discussed previously (Dessirier, *et al.*, 1999). Finally, to avoid ceiling effects at the ends of the scale, extensions with categories ranging from 16 to 30, or even higher unnumbered categories, were available at both ends of the scale on separate sheets of paper, should they be required.

2-AFC. Thirty seconds after the bilateral application of capsaicin, or 15 s after application of citric acid, subjects were asked to choose which side of the tongue gave rise to a stronger sensation (2-AFC) (Green and Swets, 1966). Use of the 2-AFC ensured sufficient sensitivity to detect small and potentially confusable intensity differences between the two sides of the tongue. This also enabled d' values to be computed to measure the magnitude of the difference. However, should the differences be perfectly discriminable, ratio scale measures like d' tend to infinity and are thus inappropriate. Accordingly, subjects also gave ratings of the perceived intensity of irritation on each side separately using a unipolar category scale with 0 (no sensation) at one end and 10 (intense irritation) at the other end, as in the first 'matching' session. Another advantage of intensity ratings was to provide an estimate of the remaining sensation on the treated side, thus documenting whether prior stimulation had partially or completely inhibited the irritant sensation. Here, the 0–10 unipolar scale was used rather than the bipolar scale because the goal was to compare intensities on the two sides of the tongue rather than to record relative increases or decreases in intensity during repeated stimulation. The 2-AFC and rating tasks were performed at 30 s intervals for a total time of 3 min following the initial application. The filter papers were left on the tongue for the entire 3 min period.

Statistical analysis

The sequential stimulation data were analyzed using analysis of variance (ANOVA) and LSD (Fisher's least significant difference) *post hoc* tests. To perform the ANOVA, the ratings were transformed first by allocating a positive sign to the scores corresponding to categories on the right side of the scale (sensations stronger compared with the initial sensation) and a negative sign to the number corresponding to categories on the left side of the scale (sensations weaker than the initial one). Then, the scale was renumbered to avoid distortion, by restoring 0 to the center of the scale, rather than unity.

The self- and cross-desensitization parts of the experiment were analyzed using a d' analysis (Ennis, 1993; Bi *et al.*, 1995) with an additional binomial analysis for the 2-AFC results and Student's *t*-test for the intensity ratings.

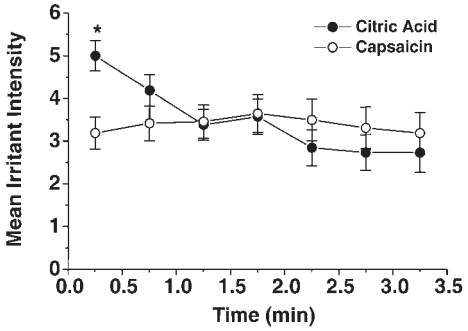

Figure 1 Approximate matching of the intensity of irritation elicited by capsaicin and citric acid. The graph plots mean irritation intensity ratings for the capsaicin- (3 ppm) and citric acid-stimulated (250 mM) sides of the tongue versus time following stimulus onset. Error bars indicate the SE. Asterisks indicate a significant difference between the two sides ($P < 0.05$, *t*-test).

Estimates of overdispersion were not possible because each subject performed each experiment a single time. For all significant differences reported $P < 0.05$.

Results and discussion

Approximate matching between capsaicin and citric acid

When performing the 2-AFC test 15 s after application of the capsaicin solution on one side of the tongue and the citric acid solution on the other side, a significant majority of subjects (22/26, binomial, $P = 0.001$) perceived the citric acid-evoked sensation to be stronger. In addition, the mean intensity rating was significantly higher on the citric acid-stimulated side (5.0 versus 3.2, *t*-test, $P = 0.002$; see Figure 1). This demonstrated that the two solutions were not matched for intensity of evoked irritation at the beginning of the stimulation. However, the irritation intensity induced by citric acid decreased to become virtually identical to that of capsaicin and then weaker, albeit not significantly (Figure 1). This formally confirmed the difference in the time course of irritation that was noted in pilot tests, with citric acid inducing a short-lived irritation while capsaicin evoked irritation that persisted for the entire 3 min testing period. Finally, a comparison between the maximum mean ratings for each compound (citric acid at 15 s and capsaicin at 105 s) revealed a significant difference (5.0 versus 3.7, *t*-test, $P = 0.03$), indicating a stronger sensory impact of citric acid than capsaicin. Approximate matching was undertaken primarily for the investigation of mutual desensitization effects, to rule out the possibility that one chemical might cross-desensitize the other but not vice versa, merely because it elicits an overwhelmingly larger sensation. For the present purposes, capsaicin cross-desensitization to citric acid irritation has already been established (Gilmore and Green, 1993; Dessirier *et al.*, 2000a), while the reverse has not. It was therefore preferable to err on the side of stronger irritation from citric acid to

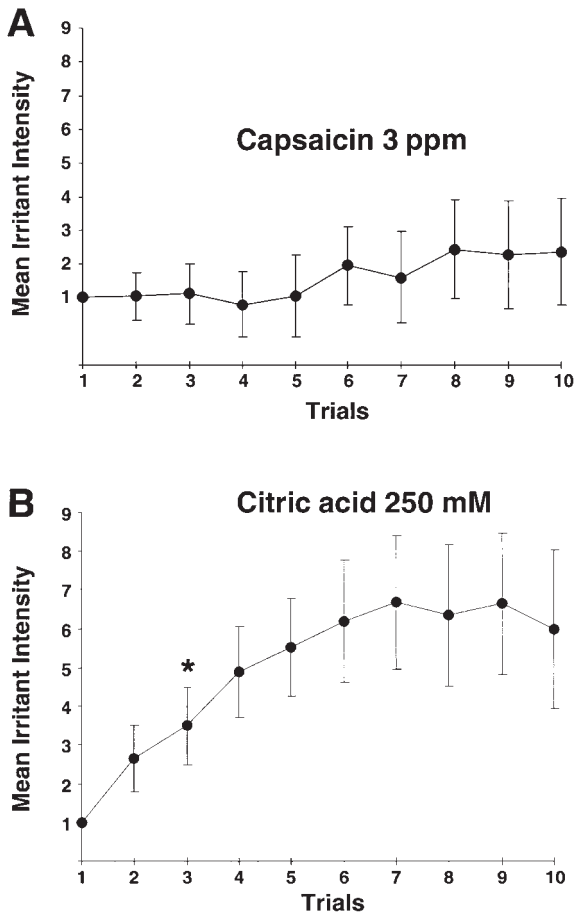

Figure 2 Sensitization by citric acid but not capsaicin. **(A)** Capsaicin. The graph plots mean irritation intensity reported 25 s following stimulus onset as a function of trials of capsaicin (3 ppm) application at 1 min ISI ($n = 26$ subjects). Error bars indicate the SE. **(B)** Citric acid. Graph as in (A) plotting mean irritation intensity versus trial of citric acid (250 mM) application ($n = 26$ subjects). The asterisk indicates the first significant increase from the initial rating ($P < 0.05$, ANOVA with LSD *post hoc* test).

obviate any possible masking effect by strong capsaicin irritation.

Response to repeated stimulation with capsaicin

On average there was no significant increase in ratings of irritation elicited by repeated application of capsaicin in both sessions separately (ANOVA with subjects and trials as factors, $P > 0.5$) or combined (ANOVA with subjects, trials and sessions as factors, $P > 0.5$) (Figure 2A). There was a high degree of inter-individual variability, with some subjects exhibiting strong sensitization and others showing no change across trials, with an average non-significant tendency toward a slight sensitization (Figure 2A). There was also high intra-individual variability, with only six subjects exhibiting consistent sensitization and four others exhibiting consistent desensitization over two sessions. The criteria for sensitization and desensitization were that four of the five last ratings had to be significantly above (for sensitization) or below (desensitization) the starting value of 1 (i.e. four of the five last ratings were more than one LSD value above or below the first rating of 1). The present lack of significant capsaicin sensitization when applied at 1 min intervals is at odds with some previous reports (Green, 1989; Dessirier *et al.*, 1997) but consistent with other studies using the same application paradigm (Green, 1996; Green and Rentmeister-Bryant, 1998). A more clear cut sensitization has been reported with capsaicin applied sequentially at shorter (5 and 15 s) ISIs (Green and Rentmeister-Bryant, 1998).

Discovery of SIR by Green (Green, 1996) raises the possibility that sensitization might reflect a recovery from prior desensitization induced by capsaicin consumption. The high inter- and intra-individual variability would then result from consumption of capsaicin or other desensitizing agents prior to some experimental sessions but not others. However, high variability has been consistently observed despite efforts to control for dietary exposure to capsaicin and other irritants (Dessirier *et al.*, 1997, 1999). There was little if any correlation between subjects' self-reported intake of capsaicin-containing foods and response patterns to sequential stimulation by irritant chemicals (Green, 1996; Prescott, 1996; McBurney, 1997) (Dessirier *et al.*, unpublished observations). Furthermore, SIR was observed in facial skin, which is unlikely to have been previously exposed to capsaicin (Green and Rentmeister-Bryant, 1998). Thus, the shape of the curve of intensity ratings across trials (Figure 2A) depends on the proportion of subjects that reliably exhibit sensitization or desensitization, with possible influences of prior dietary consumption of irritant-containing products and other unknown factors.

Response to repeated stimulation with citric acid

Upon repeated stimulation with citric acid at 1 min intervals the average intensity rating for irritation increased significantly from the first to the tenth application in both sessions separately (ANOVA with subjects and trials as factors, $P < 0.001$) or combined (ANOVA with subjects, trials and sessions as factors, $P < 0.001$). The mean ratings during the 'cit–cap' session are presented in Figure 2B. Despite some variability, sensitization during repeated stimulation with citric acid was more consistent both inter- and intra-individually than for capsaicin. Thus, 12 of 26 subjects consistently showed sensitization over the two sessions, while only one subject consistently exhibited desensitization. In addition, in the 'cit–cit' session 14 subjects were sensitized while three were desensitized (Figure 2B) and in the 'cit–cap' session 16 were sensitized and four were desensitized. We conclude that citric acid exhibits sensitization during recurrent stimulation at 1 min intervals, as previously reported for capsaicin and piperine (Green, 1989; Dessirier *et al.*, 1997, 1999) as well as NaCl and KCl at molar concentrations (Green and Gelhard, 1989). Many other irritant compounds tested to date (nicotine, menthol and zingerone; see Introduction) exhibit a progressive

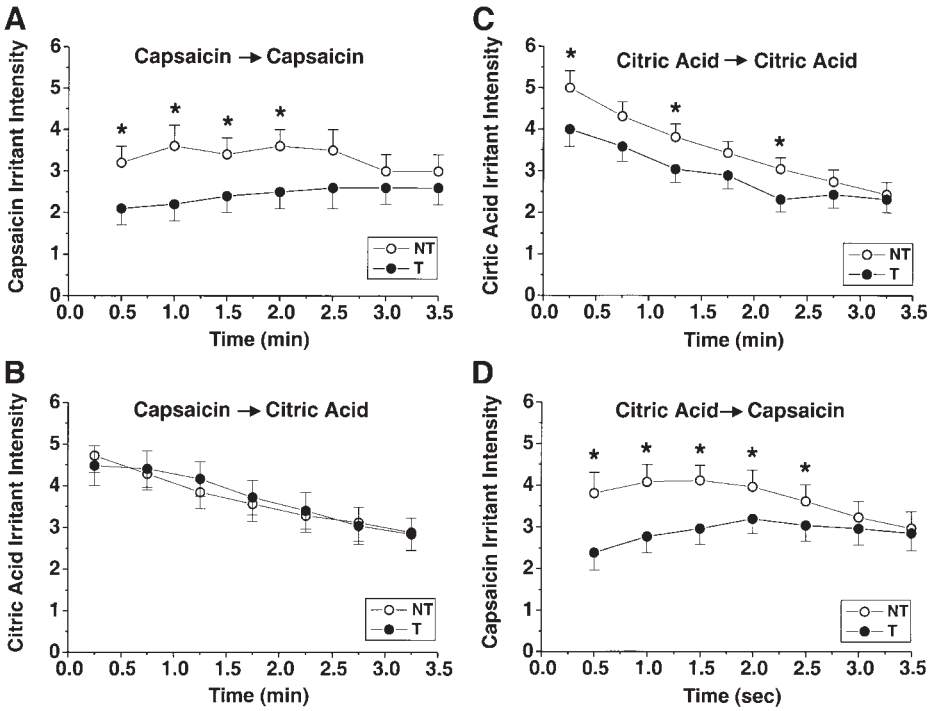

Figure 3 Self- and cross-desensitization. **(A)** Effect of capsaicin pretreatment on irritation evoked by capsaicin (capsaicin self-desensitization). The graph plots the mean irritation intensity for the capsaicin pretreated (T, filled circles) and non-treated (NT, open circles) side of the tongue versus time following bilateral application of capsaicin. Note the significant self-desensitization. Error bars indicate the SE. Asterisks indicate significant differences between T and NT ($P < 0.05$, *t*-test). **(B)** Effect of capsaicin pretreatment on irritation evoked by citric acid (capsaicin cross-desensitization of citric acid). Graph as in (A) plotting mean ratings for capsaicin pretreated and non-treated sides following bilateral application of citric acid. Note the absence of cross-desensitization. **(C)** Effect of citric acid pretreatment on irritation evoked by citric acid (citric acid self-desensitization) [format as in (A)]. Note the significant self-desensitization by citric acid. **(D)** Effect of citric acid pretreatment on irritation evoked by capsaicin (citric acid cross-desensitization of capsaicin). Note that pretreatment with citric acid induced significant cross-desensitization of the irritation elicited by subsequent capsaicin.

desensitization during repeated stimulations at the ISI tested.

Capsaicin self-desensitization

Consistent with previous results, capsaicin exhibited self-desensitization. Thus, when capsaicin was applied bilaterally following 10 sequential unilateral stimuli and a 10 min rest period, a significant majority of subjects (19/26, binomial, $P = 0.029$) indicated that the previously unstimulated side yielded a stronger irritant sensation. The corresponding group d' value was 0.87 and was significantly different from 0 ($P = 0.014$). Further, the mean rating on that side was significantly larger than on the previously stimulated side (3.1 versus 2.1, *t*-test, $P = 0.021$; Figure 3A). The difference remained significant until 2 min post-application, after which it was no longer significant (Figure 3A).

Capsaicin cross-desensitization to citric acid irritation

Capsaicin did not cross-desensitize irritation elicited by citric acid. Thus, 15 s after bilateral application of citric acid, only 15/26 subjects (binomial, $P = 0.55$) indicated the previously non-stimulated side as yielding a stronger irritation. This corresponded to a group d' value of 0.27, which is not significantly different from 0 ($P = 0.22$). The difference between the mean ratings on the two sides was not significant (4.7 versus 4.5, *t*-test, $P = 0.38$) and remained so for the entire 3 min testing period (Figure 3B).

This result is not consistent with previous results showing a cross-desensitization effect of capsaicin treatment on citric acid-evoked irritation (Geppetti *et al.*, 1993; Gilmore and Green, 1993; Dessirier *et al.*, 2000a). However, our finding is consistent with one study which also failed to show significant cross-desensitization of citric acid-evoked irritation by capsaicin (Green, 1996). One possible explanation is that the capsaicin solution used here (3 ppm, 10 μM) was too weak to induce detectable cross-desensitization. Indeed, studies reporting significant cross-desensitization of acid-evoked irritation by capsaicin used a higher (10 ppm, 33 μM) capsaicin concentration (Gilmore and Green, 1993) or dose (Dessirier *et al.*, 2000), while the study reporting an absence of cross-desensitization used a lower (5 ppm, 17 μM) capsaicin concentration (Green, 1996). One speculation is that only a relatively small proportion of trigeminal fibers mediating oral irritation induced by acidic stimuli are capsaicin-sensitive. Only 50% of corneal nociceptors that were excited by CO_2 also responded to capsaicin (Chen *et al.*, 1997). This would imply that stronger capsaicin treatment is needed to achieve detectable cross-desensitization. Finally, the citric acid solution used in this study yielded a

fairly strong irritant sensation, which might have rendered the detection of a small difference between the two sides of the tongue more difficult, consistent with a Weber's law effect.

Citric acid self-desensitization

Stimulation with citric acid followed by a 10 min rest period induced a significant reduction in the sensation elicited by subsequent citric acid stimulation: self-desensitization. Thus, 15 s after bilateral application of citric acid, a significant majority of subjects (19/26, binomial, $P = 0.029$) chose the previously non-stimulated side of the tongue as yielding a stronger sensation. This corresponded to a significant group d' value of 0.87 ($P = 0.014$). The mean ratings between the two sides were also significantly different (5.0 versus 4.0, t-test, $P = 0.009$). This difference remained significant for 2 min after the initial application (Figure 3C). Thus, like all other irritant chemicals tested to date, citric acid elicits self-desensitization, which may be an inherent property of chemesthetic agents in general.

Citric acid cross-desensitization to capsaicin

Stimulation with citric acid followed by a rest period also led to a decrease in the capsaicin-evoked irritation: cross-desensitization. Thus, 30 s after capsaicin was applied bilaterally the side of the tongue that had not been stimulated with citric acid was chosen as having stronger irritation by a significant majority of subjects (21/26, binomial test, $P = 0.002$). This corresponded to a significant group d' value of 1.23 ($P = 0.003$) and the mean rating on the previously non-stimulated side was significantly stronger (3.8 versus 2.4, t-test, $P < 0.001$; Figure 3D).

The significance of this finding is that citric acid possesses the ability to cross-desensitize the sensation elicited by other irritant chemicals, a property previously attributed only to the vanilloids capsaicin and piperine. This and sensitization are properties that citric acid has in common with capsaicin and piperine, thus indirectly supporting the involvement of vanilloid receptors in acid-evoked irritation. A second experiment was therefore performed to determine whether citric acid induces cross-SIR from capsaicin desensitization, another property typical of vanilloids.

Experiment II

Materials and methods

Subjects

Twenty healthy individuals (two males, 18 females, aged 18–26 years), students and staff at the University of California at Davis, volunteered to participate in the study. None had participated in Experiment I. All refrained from eating or drinking for at least 1 h prior to each experimental session and from eating spicy food for the preceding 2 days.

Chemical stimuli and application procedure

A stronger capsaicin solution (33 ppm, 110 μM) than was used in Experiment I was prepared by diluting the 0.1% capsaicin stock solution used in Experiment I. The same 250 mM citric acid solution was used.

Capsaicin was applied to one side of the tongue and dH_2O to the other side using cotton swabs (Puritan; Hardwood Product Co., Guilford, ME). The side of the tongue receiving capsaicin was counter-balanced across subjects. The cotton swabs were rolled on the dorsum of the tongue from the tip towards the back of the mouth and back in one stroke. The first stroke was made on each side of the midline, the second stroke approximately midway between the midline and the sides of the tongue and the last stroke close to the sides. This was repeated three times. Five minutes after the treatment subjects were asked to compare the remaining irritation on both sides of the tongue (2-AFC) and to rate the intensity of the sensation on each side separately, as described in Experiment I. These tests were repeated 10 and 15 min after treatment. If any of the ratings obtained at 15 min were 2 or more, the tests were repeated every 5 min until both ratings dropped to 1 or less. This ensured that the sensation induced by capsaicin had ceased and that desensitization had occurred before proceeding. The rationale is that absence of sensation is a decision prone to response bias [criterion shift (O'Mahony, 1992)], so that bilateral ratings of 0 might not be given for some time even after the sensation has actually vanished.

To test whether the stronger capsaicin concentration had effectively desensitized the citric acid-evoked irritation, two large filter paper disks were soaked with 40 μl of 250 mM citric acid and placed on each side of the tongue. Fifteen seconds after application subjects were asked to perform the same 2-AFC and rating procedures.

Large filter paper disks soaked with 40 μl of citric acid solution were then repeatedly applied to the capsaicin-treated side of the tongue 10 times at 1 min intervals (30 sec ISI) as in the sensitization procedure in Experiment I. Subjects were similarly asked to provide intensity ratings 15 s after each filter paper application using the bipolar scale described in Experiment I. Subjects were allowed to use the suction device as before. Data were analyzed as in Experiment I.

Results and discussion

Treatment-evoked sensation

Application of capsaicin (33 ppm) to one side of the tongue elicited a significant irritant sensation on the capsaicin-treated side that subsided within 15–20 min. Thus, at 5 min post-treatment all subjects (20/20, binomial, $P < 0.001$) chose the treated side as stronger and the mean rating on the capsaicin-treated side was significantly greater (5.2 versus 1.2, t-test, $P = 0.002$). At 15 min post-treatment all subjects still chose the capsaicin-treated side as stronger, but the intensity rating was no longer significantly greater on that

side. Only six subjects required 20 min post-treatment until intensity ratings on both sides were 1 or less.

Capsaicin cross-desensitization to citric acid

When citric acid was applied bilaterally after capsaicin-evoked irritation had ceased, 18/20 subjects chose the capsaicin non-treated side as yielding a stronger irritation sensation (binomial, $P < 0.001$). This corresponded to a significant group d' value of 1.81 ($P = 0.002$). The mean rating on the capsaicin non-treated side was also significantly higher (5.0 versus. 3.2, *t*-test, $P < 0.001$). This indicates that the capsaicin treatment had successfully desensitized that side of the tongue to citric acid-evoked irritation. This result also supports our contention that the failure to establish cross-desensitization in Experiment I was because the 3 ppm capsaicin concentration was too weak even when given 10 times in succession. It is, however, interesting to note that a single application of the ~10-fold higher capsaicin concentration induced significant cross-desensitization, suggesting that the total dose of capsaicin is less important for desensitization than its maximal sensory impact.

Repeated citric acid stimulation

When citric acid was subsequently applied sequentially to the capsaicin-desensitized side of the tongue, irritation ratings increased significantly across trials (ANOVA with subjects and trials as factors, $P < 0.001$). Thus, all intensity ratings from the third stimulus were significantly higher than the initial rating. Despite inter-individual variability, a significant majority of subjects (15/20, binomial, $P = 0.041$) showed a clear increase in the perceived irritation (Figure 4). This is consistent with the phenomenon of cross-SIR established between capsaicin and piperine (Green, 1996). In the same study, cross-SIR from capsaicin desensitization did not occur with three consecutive applications of 125 mM citric acid. The present ability to induce cross-SIR might relate to the higher concentration of citric acid used.

The present results thus show that in addition to exhibiting sensitization, self-desensitization and cross-desensitization to capsaicin, citric acid can also induce cross-SIR from capsaicin desensitization. Our results lend further support to the link among sensitization, cross-desensitization and SIR noted earlier (Green, 1996). Only piperine had previously been shown to exhibit cross-desensitization to and cross-SIR with capsaicin (Green, 1996), and the similar properties shown presently for citric acid provide additional indirect support for potential involvement of a vanilloid receptor in acid-evoked irritation on the tongue.

The present results have possible clinical implications. Despite concerns regarding its true efficacy and the difficulty of running blind clinical trials because of the obvious burning sensation perceived during treatment (Carter, 1991; Watson, 1994), capsaicin desensitization via commercially available creams is nonetheless used increasingly to treat pain arising from arthritis and other inflammatory conditions (Freidrich, 1988; Carter, 1991; Deal *et al.*, 1991; Zhang and Li Wan Po, 1994; Berger *et al.*, 1995). Because inflammation, along with the release of various inflammatory mediators, induces tissue acidification (Reeh and Steen, 1996), the ability of protons to induce recovery (cross-SIR) from capsaicin desensitization may potentiate irritation and pain and reduce the analgesic efficacy of capsaicin.

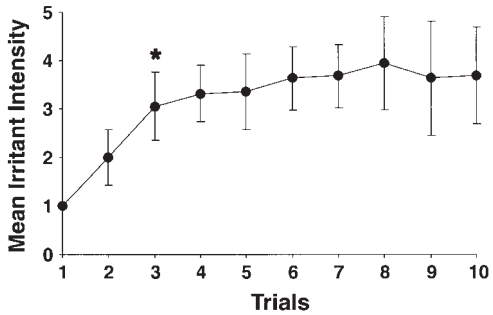

Figure 4 Sensitization with repeated stimulation by citric acid following capsaicin desensitization (cross-SIR). Format as in Figure 2B. The graph plots mean irritation intensity versus trial of citric acid (250 mM) application (n = 20 subjects).

Experiment III

Besides the possible involvement of a vanilloid receptor, acid-evoked discomfort is thought to involve activation of another class of receptors, members of the amiloride-sensitive Na^+ channel/degenerin family (ASIC) [for a review see (Waldmann and Lazdunski, 1998)]. It is thus possible that citric acid-evoked irritation on the tongue involves activation of ASICs and would be inhibited by amiloride. Amiloride has also been shown to inhibit acid-evoked inward currents in hamster taste cells thought to result from influx of protons through amiloride-sensitive Na^+ channels (Gilbertson *et al.*, 1992) and also reduces aversiveness to citric acid in hamster taste preference tests (Gilbertson and Gilbertson, 1994). However, in humans amiloride appears only to reduce the gustatory perception of NaCl (Schiffman *et al.*, 1983) but not KCl or citric acid (Ossebaard and Smith, 1995; Smith and Ossebaard, 1995; Tennissen and McCutcheon, 1996), suggesting species differences. The effect of amiloride on irritation or pain elicited by irritant chemicals in humans has not previously been tested. In the rat, however, amiloride, given systemically, intrathecally or intracranially, reduced nocifensive behavioral responses induced by capsaicin, acetic acid and formalin but not noxious heat (Ferreira *et al.*, 1999), pointing to a potential action of amiloride on pain of chemogenic origin. Amiloride blockade of voltage-gated Na^+ and Ca^{2+} channels was postulated to explain these effects. In the final experiment we therefore tested whether amiloride affects irritation elicited by citric acid, as predicted by involvement of ASIC. To control for a more general anti-nociceptive action, we also tested the effect of amiloride on capsaicin-evoked irritation.

Materials and methods

Subjects

Forty healthy individuals (12 males, 28 females, aged 18–43 years), students and staff at the University of California at Davis, volunteered to participate in the study. As in Experiments I and II, all refrained from eating or drinking for at least 1 h prior to each experimental session and from eating spicy food for 2 days prior to testing.

Chemical stimuli

Amiloride (Sigma) was dissolved in dH_2O to a concentration of 1 mM and the same citric acid (250 mM) and capsaicin (3 ppm, 10 μM) solutions were prepared as in Experiment I.

Application and rating procedures

Amiloride was applied to one side of the tongue while dH_2O was applied to the other side using cotton swabs in the same manner as described in Experiment II for capsaicin. The amiloride-treated side was counter-balanced across subjects. Subjects then rested with their mouth closed for 1 min, after which the same treatment was repeated. One minute later, 40 μl of the citric acid solution was pipetted onto two large filter paper disks (176.7 mm^2; Whatmann). The two filter paper disks were then placed one on each side of the tongue at the locations treated with amiloride and dH_2O and subjects then performed the 2-AFC test and gave intensity ratings as described in Experiments I and II.

The effect of amiloride on capsaicin-evoked irritation was also studied. Thus, the 40 subjects tested in the first part of the experiment with citric acid also participated in a second session that was identical except that a 3 ppm capsaicin solution was used instead of citric acid. As in Experiment I, filter papers were air dried after pipetting capsaicin to eliminate any effect of ethanol and soaked with the same volume of distilled water immediately prior to application. The side receiving amiloride was counter-balanced across subjects.

As in Experiments I and II, subjects used a suction device. Data were analyzed as in Experiments I and II.

Results and discussion

Pretreatment with amiloride led to a weak but significant reduction in irritation induced by citric acid. Thus, when citric acid was applied bilaterally after unilateral amiloride treatment the untreated side was chosen as yielding a stronger sensation by a significant majority of subjects (27/40, binomial, $P = 0.038$). This corresponded to a small but significant group d' of 0.64 ($P = 0.017$). In addition, the mean intensity ratings on each side were significantly different (5.5 versus 4.8, t-test, $P = 0.02$; Figure 5A). It is possible that part of the effect of amiloride may have been due to a reduction in the taste (e.g. sourness) sensation that subjects incorporated into their overall rating of irritation, despite being instructed to ignore the taste component.

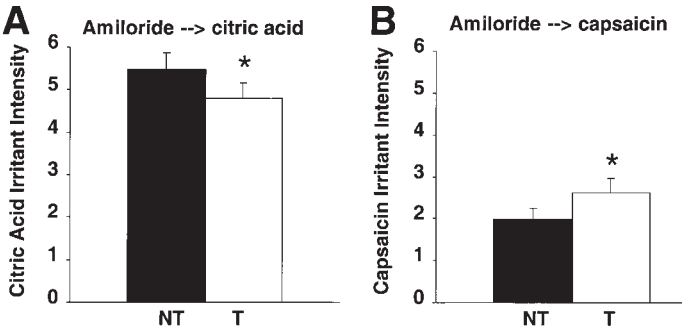

Figure 5 Amiloride effects on citric acid- and capsaicin-induced irritation. **(A)** Amiloride effect on citric acid-induced irritation. The bar graph plots mean intensity ratings on the amiloride pretreated (T, open bar) and non-treated (NT, filled bar) sides of the tongue. Note the small but significant reduction on the side receiving amiloride. Error bars indicate the SE. Asterisks over open bars indicate significant differences between T and NT ($P < 0.05$, t-test). **(B)** Amiloride effect on irritation evoked by capsaicin [format as in (A)]. Note the small but significant increase on the side receiving amiloride.

However, this is unlikely since it was previously reported that amiloride had no effect on the sour taste elicited by citric acid (Tennissen and McCutcheon, 1996). The present results thus indicate the potential involvement of an amiloride-sensitive mechanism in acid-induced oral irritation.

In contrast, amiloride did not reduce capsaicin-evoked irritation, but instead resulted in a small but significant enhancement of irritation on the amiloride-treated side. Thus, when capsaicin was applied bilaterally following unilateral treatment with amiloride, 28/40 subjects chose the amiloride-treated side as yielding a stronger sensation (binomial, $P = 0.008$; group $d' = 0.78$, $P = 0.007$). The mean intensity ratings on each side of the tongue were significantly different (2.0 versus 2.6, t-test, $P = 0.008$; Figure 5B).

That amiloride potentiated capsaicin-evoked irritation argues against a general anti-nociceptive action of amiloride, which was suggested by the finding that amiloride suppressed capsaicin- and formalin-induced licking in mice (Ferreira *et al.*, 1999). Our results also argue against the possibility that amiloride inhibits the vanilloid receptor. It was previously shown that amiloride enhanced responses of cutaneous nociceptors to acidic stimulation with a saturated CO_2 solution (Steen *et al.*, 1999). These authors proposed a mechanism whereby amiloride blocked a Na^+/H^+ exchange pump in the terminal endings of nociceptors, preventing extrusion of protons and thus lowering intracellular pH. Conceivably, in the present study blockage of the Na^+/H^+ exchange pump might have led to an increase in the intracellular proton concentration, thereby potentiating capsaicin-evoked depolarization of the nociceptor endings (Bevan and Geppetti, 1994).

General discussion

The present study provides evidence that citric acid has

sensory properties similar to those exhibited by capsaicin: sensitization at short intervals, self- and cross-desensitization at longer (10 min) intervals and cross-SIR when re-applied at short intervals following capsaicin desensitization. These findings are consistent with the hypothesis that acidic stimuli stimulate the chemesthetic sense via activation of a subtype of the vanilloid receptor such as VR1 (Caterina *et al.*, 1997). This is further supported by a recent study showing that sensory neurons originating from VR1 knock-out mice were virtually insensitive to acidic stimuli (Caterina *et al.*, 2000). Acid stimulation of VR1 receptors might be achieved if acidification lowered the threshold for noxious heat activation of VR1 to body temperature (Tominaga *et al.*, 1998). Indeed, acidification to pH 6.4 was shown to activate VR1 at 37°C and such a pH might be readily achieved in the lingual tissue with application of 250 mM citric acid, which has a pH of ~2. Nonetheless, involvement of vanilloid receptors does not necessarily confer properties of cross-desensitization or cross-SIR in all cases. Zingerone induced inward currents in trigeminal ganglion neurons in a manner that was reduced by the vanilloid antagonist capsazepine and appeared to cross-desensitize the effects of capsaicin and piperine (Liu and Simon, 1996). However, in animal behavioral studies and human psychophysical experiments, zingerone exhibited neither cross-desensitization effects (Szolcsányi and Jancsó-Gábor, 1976; Gilmore and Green, 1993) nor cross-SIR following capsaicin desensitization (Green, 1996). Thus, the nature of the receptor is not the only factor determining the sensory properties of sensitization, cross-desensitization and cross-SIR, and other factors such as kinetics of agonist binding, activation and desensitization at the receptor level may be as important [for a discussion see (Liu and Simon, 1996)].

The last experiment in this study points to another, complementary amiloride-sensitive mechanism for acidic stimulation of nociceptors such as ASIC (Waldmann and Lazdunski, 1998), although passive influx of protons through an amiloride-sensitive Na^+ channel, as proposed for sour taste (Kinnamon and Margolskee, 1996), cannot be excluded. Nevertheless, it must be kept in mind that the presently observed inhibitory effect of amiloride on citric acid-evoked irritation was weak, indicating that the irritation is mediated largely through an amiloride-insensitive mechanism. Besides acidic potentiation of VR1, other possible amiloride-insensitive mechanisms include activation of ligand-gated channels resembling ASIC3 (DRASIC), an ASIC isoform found in dorsal root ganglion neurons exhibiting a pH-dependent sustained current that is not inhibited by amiloride (Waldmann *et al.*, 1997a).

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