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Self- and Cross-desensitization of Oral Irritation by Menthol and Cinnamaldehyde (CA) via Peripheral Interactions at Trigeminal Sensory Neurons

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Abstract

Menthol and cinnamaldehyde (CA) are plant-derived spices commonly used in oral hygiene products, chewing gum, and many other applications. However, little is known regarding their sensory interactions in the oral cavity. We used a human psychophysics approach to investigate the temporal dynamics of oral irritation elicited by sequential application of menthol and/or CA, and ratiometric calcium imaging methods to investigate activation of rat trigeminal ganglion (TG) cells by these agents. Irritancy decreased significantly with sequential oral application of menthol and CA (self-desensitization). Menthol cross-desensitized irritation elicited by CA, and vice versa, over a time course of at least 60 min. Seventeen and 19% of TG cells were activated by menthol and CA, respectively, with ~50% responding to both. TG cells exhibited significant self-desensitization to menthol applied at a 5, but not 10, min interval. They also exhibited significant self-desensitization to CA at 400 but not 200 μM. Menthol cross-desensitized TG cell responses to CA. CA at a concentration of 400 but not 200 μM also cross-desensitized menthol-evoked responses. The results support the argument that the perceived reductions in oral irritancy and cross-interactions between menthol and CA and menthol observed (at least at short interstimulus intervals) can be largely accounted for by the properties of trigeminal sensory neurons innervating the tongue.

Key words: cinnamaldehyde, desensitization, menthol, oral irritation, psychophysics, trigeminal ganglion cell

Introduction

Menthol and cinnamaldehyde (CA) are plant-derived spices used in a variety of cuisines and are also widely used additives in oral hygiene products, chewing gum, and many other products. At higher concentrations, both chemicals induce an oral irritant sensation that decreased in magnitude (self-desensitization) upon repeated application at short (1-min) interstimulus intervals (Cliff and Green 1994, 1996; Prescott and Swain-Campbell 2000; Dessirier et al. 2001). When applied topically to the skin, menthol elicited cold pain (Wasner et al. 2004; Green and Schoen 2007), whereas CA elicited burning pain (Namer et al. 2005). Despite the widespread commercial use of these agents, little is known regarding their interaction in the oral cavity. Menthol acts at the thermosensitive transient receptor potential (TRP) channel TRPM8 that is sensitive to temperature decreases in the innocuous range (McKemy et al. 2002; Peier et al. 2002). TRPM8 is expressed in innocuous cold fibers and also appears to be coexpressed with TRPV1 in nociceptors (McKemy et al. 2002; Reid et al. 2002; Viana et al. 2002; Xing et al. 2006; Belmonte et al. 2009), potentially explaining why oral menthol elicits both innocuous cooling and irritant sensations (Cliff and Green 1994, 1996; Dessirier et al. 2001). In contrast, CA acts at TRPA1 (Story et al. 2003; Jordt et al. 2004) that is coexpressed with TRPV1 in nociceptors (Story et al. 2003). Intraoral application of both menthol and CA excite cold-sensitive trigeminal subnucleus caudalis (Vc) neurons in rats in a manner exhibiting self-desensitization (Carstens et al. 2005; Zanotto et al. 2007) and reciprocal cross-desensitization (Zanotto et al. 2008).

In the present human psychophysical study, we wished to investigate the temporal dynamics of oral irritation elicited by menthol and CA and their interactions, given that these

agents are experienced by many people on a daily basis. We hypothesized that the oral irritation, but not cooling sensation (Cliff and Green 1994, 1996), elicited by menthol would exhibit self-desensitization across repeated trials as well as cross-desensitization by CA. We similarly hypothesized that CA-evoked oral irritation would exhibit self-desensitization similar to another TRPA1 agonist, mustard oil (Simons et al. 2003), as well as cross-desensitization by menthol (Zanotto et al. 2008). Two other commonly encountered oral irritants, nicotine and capsaicin, exhibited self-desensitization that persisted for hours or days (Carstens et al. 2007). We presently wished to determine if menthol and CA similarly exhibit prolonged self-desensitization, with implications for the everyday sensory experiences associated with oral hygiene products, chewing gum, and other ingested substances that contain these agents.

A second aim of the present study was to investigate if the temporal dynamics and cross-interactions of menthol and CA assessed perceptually can be explained by effects of these compounds peripherally at trigeminal nerve endings. To this end, we also investigated the effects of menthol and CA on trigeminal primary sensory neurons using the method of calcium imaging of cultured rat trigeminal ganglion (TG) cells. We hypothesized that the responses of TG cells to menthol and CA would exhibit self- and reciprocal cross-desensitization, similar to the predicted psychophysical effects.

Methods

Psychophysical testing

Data were obtained from a population of 169 subjects (117 females and 52 males) consisting of students, staff, and faculty members at University of California Davis under a protocol approved by the University of California Davis Human Subjects Committee. All subjects were required to sign an informed consent form. They were instructed not to eat spicy food for 3 days prior to their participation.

The approach was similar to that employed in our previous studies (Simons et al. 2003; Carstens et al. 2007). Each experimental session involved application of either menthol (19 or 29 mM; Givaudan Flavors Corp.) or CA (15 or 30 mM; Sigma Chemical Co.) onto one side of the dorsal anterior tongue by filter paper (1.5 cm diameter) wetted with 40 µl of the chemical, after which the mouth was closed. Simultaneously, vehicle (4% ethanol/1% Tween) was applied in an identical manner by filter paper to a corresponding location on the opposite side of the tongue. Both filter papers were removed after 30 s. For studies of cross-desensitization, the concentrations of menthol and CA were matched according to sensory intensity. This was accomplished in pilot studies by applying 1 filter paper wetted with menthol and another wetted with CA, simultaneously on each side of the tongue and having subjects state on which side they experienced stronger irritation. Menthol concentrations of 19 and 29 mM approximately matched CA at 15 and 30 mM, in that subjects did not reliably choose one side over the other as having stronger irritation. After the initial chemical application, a waiting period of 5, 30, or 60 min ensued, followed by bilateral application of the test chemical (either menthol or CA) within the pretreated tongue areas using smaller (1 cm diameter) filter papers wetted with 20 µl of the chemical (see Figure 1A). The various combinations of sequential chemical applications are summarized in Table 1.

After the interstimulus interval and bilateral application of the test chemical, subjects were asked to state on which side of the tongue they experienced stronger irritation in a 2-alternative forced-choice (2-AFC) design. Immediately following the 2-AFC, subjects were asked to independently rate the intensity of irritation on each side of the tongue using the general labeled magnitude (gLMS) scale (Bartoshuk et al. 2004). Subjects were provided a sheet with 2 gLMS scales for the 2 sides of the tongue and marked the site on the scale that corresponded to their intensity rating. The gLMS contains verbal descriptors (no sensation, barely detectable, weak, moderate, strong, very strong, and strongest imaginable) spaced in a roughly logarithmic manner along a vertical scale (Green et al. 1993). Subjects received instructions on how to use the gLMS at the beginning of the experimental session.

The 2-AFC data were submitted to a binomial test to establish if a significant proportion of subjects consistently chose the vehicle-pretreated side of the tongue as having stronger irritation, with $P < 0.05$ considered to be statistically significant. For this reason, a minimum of 30 subjects were tested under each experimental condition because the binomial distribution approaches a normal distribution at $n = 30$. Many subjects participated in more than one experimental condition. For gLMS ratings, the distance of the mark from the end of the scale was measured in millimeters, a value of 1 was added to eliminate 0 scores, and data were log transformed. A paired t-test compared the magnitude ratings for the 2 sides of the tongue. For both analyses, a $P < 0.05$ was considered to be statistically significant.

Calcium imaging of TG cells

Cell culture

Both trigeminal ganglia were removed from 3-week-old (~100 g) male Sprague–Dawley rats and placed into Petri dishes containing Hanks buffered salt solution (Gibco, Invitrogen Life Sciences). Ganglia were minced with fine spring scissors and incubated in 40 µl papain (no. 3126, Worthington Biochemical Company) with 1 mg L-cysteine (Sigma) in 1.5 mL Hanks solution for 5 min in a 37 °C rocking water bath. The minced ganglia were then centrifuged at 200 g for 2 min, and media was then suctioned away. The ganglia were then incubated in 2 mg/mL collagenase type

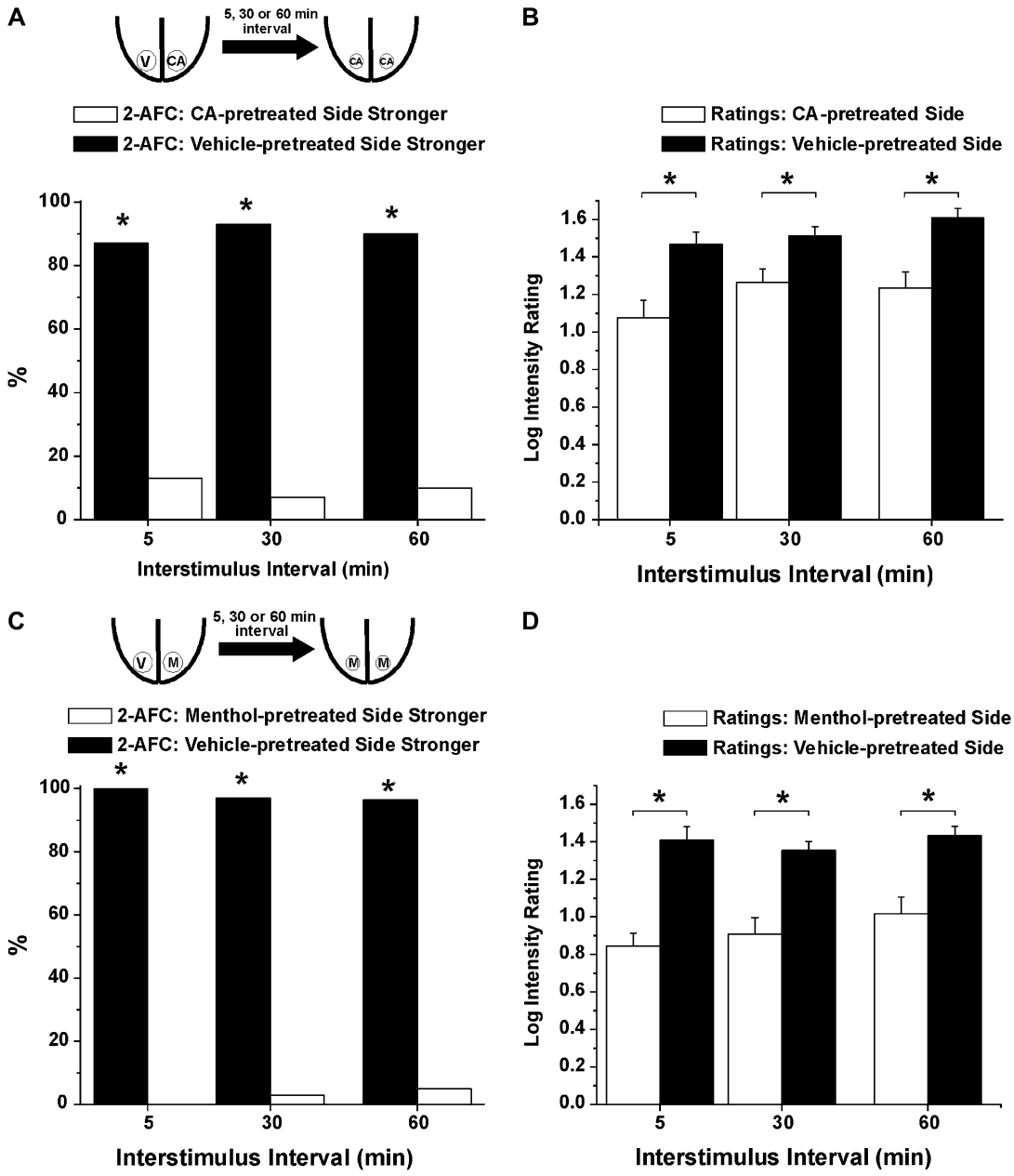

Figure 1 CA and menthol self-desensitization. **(A)** 2-AFC: CA. CA (30 mM) was applied to one half of tongue and vehicle to the other. After 5, 30, or 60 min, CA was applied to both sides. Graph plots % subjects choosing vehicle-pretreated (■) or CA-pretreated (□) side of the tongue as having stronger irritation. At all interstimulus intervals, CA evoked stronger irritation on the vehicle-pretreated side in a significant proportion of subjects, indicating CA self-desensitization. **(B)** Bilateral intensity ratings: CA. CA pretreatment significantly reduced irritation elicited by subsequent application of CA at all interstimulus intervals. **(C)** 2-AFC: menthol (19 mM) (design as in A). Graph plots percent subjects choosing vehicle-pretreated (■) or menthol-pretreated (□) side of the tongue as having stronger irritation. Asterisk indicates significant percent of subjects chose vehicle-treated side ($P < 0.05$, binomial test), indicating menthol self-desensitization. **(D)** Bilateral intensity ratings: menthol. Graph plots mean ratings for vehicle- (■) or menthol-pretreated (□) sides. Error bars: standard mean of error. Asterisk indicates significant difference between vehicle- and menthol-pretreated sides ($P < 0.05$, paired *t*-test).

II (CLS2, Worthington Biochemical Company) in Hanks solution for 5 min in a 37 °C rocking water bath and then centrifuged again at 200 g for 1 min. The minced ganglia were next triturated through polished glass pipettes with completed media consisting of Earle's minimal essential media (Gibco, Invitrogen Life Sciences) and 10% donor horse serum (Quad Five) with 1% 100× modified Eagle medium vitamin solution and penicillin–streptomycin (Gibco). The isolated TG cells were plated in 40 µl aliquots on 25-mm round glass coverslips (Bellco) coated with 1 mg/mL poly-D-lysine (Sigma) for 1 h. Cells were given 2 mL of completed media 1 h postplating and placed in a 37 °C water-jacketed

Table 1 Psychophysics experimental conditions. Each row shows the sequential application of the first chemical (unilateral application), interstimulus interval, and second chemical applied bilaterally

Condition	First chemical (unilateral application) (mM)	Interstimulus interval (min)	Second chemical (bilateral application) (mM)
1	Menthol 19	5	Menthol 19
2	Menthol 19	30	Menthol 19
3	Menthol 19	60	Menthol 19
4	Menthol 29	5	CA 30
5	Menthol 29	30	CA 30
6	CA 30	5	CA 30
7	CA 30	30	CA 30
8	CA 30	60	CA 30
9	CA 30	5	Menthol 29
10	CA 30	30	Menthol 29
11	CA 30	60	Menthol 29
12	CA 15	5	Menthol 19

CO_2-injected incubator under carbogen (5% CO_2/95% O_2), and fresh media was given after 24 h.

Imaging

TG cells were loaded with a ratiometric calcium indicator according to the manufacturer's protocol (Molecular Probes). The TG cells were incubated 1 h in 1 mM Fura 2AM dissolved in dimethyl sulfoxide (F1221, Invitrogen Life Sciences) to a final concentration of 10 μM in 5 mM glucose-supplemented Ringers solution (140 mM NaCl, 4 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, 4.54 mM NaOH, and pH adjusted to 7.4) containing 0.1% Pluronic (F127, Invitrogen Life Sciences). Cells were rinsed with Ringer's solution and allowed to rest for 10 min before being placed on a custom-made aluminum perfusion chamber and viewed through a Nikon Inverted Microscope (Eclipse TS100). Fluorescence images obtained at 340/380 nm wavelengths were viewed through a CoolSnap camera attached to a Lambda LS lamp and a Lambda 10-3 optical filter changer (Sutter Instrument Company). Ratiometric measurements were made using Simple PCI software (Compix Inc.) with an intermittent pause of 3 s between successive measurements.

Chemical stimulation

Chemical solutions were administered to one end of the perfusion chamber by a gravity fed, solenoid-controlled perfusion system (ValveLink 8.2, AutoMate Scientific). Laminar flow of chemicals over the TG cells was accomplished by vacuum suction at the opposite end of the perfusion chamber, resulting in a constant flow rate of ~2 ml/min. The following chemicals were applied: menthol (250 μM in 0.015% ethanol; Givaudan), CA (200 or 400 μM in 0.015% and 0.03% ethanol, respectively; Sigma), capsaicin (1 μM in 0.015% ethanol; Sigma), and high-K^+ Ringers (144 mM). Menthol and CA were applied for 30 or 60 s, and capsaicin for 10 s. Concentrations of menthol and CA were chosen based on previous calcium imaging studies of TG or dorsal root ganglion cells (Peier et al. 2002; Behrendt et al. 2004; Bautista et al. 2007; Hjerling-Leffler et al. 2007). These concentrations are 2–3 orders of magnitude lower than those used in the human psychophysical studies. The rationale for this difference is that in the human studies, CA and menthol applied topically must diffuse through the lingual epithelium to access nerve endings. In the cell imaging experiments, the agents have virtually immediate access to the TG cells because there is no tissue barrier, and the agents are subsequently cleared much more quickly. Separate application of vehicle (Ringers with 0.015% or 0.03% ethanol) had no effect on TG cells (data not shown).

Experimental design

For studies of self-desensitization (tachyphylaxis) either CA (200 or 400 μM) or menthol (250 μM) was the first chemical to be applied in a given experiment for 30 s, followed 5 or 10 min later by reapplication of the same chemical. The higher concentration of CA was tested because sequential application of the lower CA concentration did not result in significant self-desensitization.

Cross-desensitization between menthol and CA was assessed using 2 different paradigms. In the first paradigm, to test for CA cross-desensitization of menthol, CA (200 or 400 μM) was applied for 30 s, followed by menthol (250 μM) 5 min later. Responses to menthol post-CA were compared with responses to menthol when it was tested first in separate experiments, using an unpaired *t*-test. In the second paradigm, menthol (250 μM) was applied first for 30 s, followed 4 min later by application of CA (200 μM) for 30 s, followed 1 min later by reapplication of menthol. This sequence was also repeated using a higher concentration of CA (400 μM). This design allowed direct comparison (paired *t*-test) of menthol-evoked responses before and after application of CA. The same paradigms were similarly used to test for menthol cross-desensitization of CA. In all experiments, capsaicin was tested after the sequential applications of menthol and CA, followed lastly by high K^+.

Data analysis

Each TG cell's maximum response was taken as the highest ratio change during the 3-min poststimulus period, relative to the baseline 1 min prior to chemical application. Peak responses were normalized by subtracting the prestimulus baseline ratio. A positive response to a chemical was defined

as at least a 20% change in the baseline-corrected response. For studies of self-desensitization, peak responses (baseline corrected) to the first and second application of menthol or CA were compared by paired *t*-test. For assessment of cross-desensitization using the first paradigm, the response to CA postmenthol (or menthol post-CA) was compared to the response of cells tested with the same chemical applied first, in separate experiments, using an unpaired *t*-test. For studies of cross-desensitization using the second paradigm, TG cells were divided according to whether they responded to both menthol and CA or only to the test stimulus (menthol or CA but not both). Baseline-corrected peak responses to the first and second application of the test chemical were compared by paired *t*-test. To determine if the conditioning (cross-desensitizing) chemical produced any greater effect than self-desensitization, responses to the second application of the test chemical (after application of the cross-desensitizing chemical) were compared to responses to the second application of the same test chemical (with no intervening application of the other chemical) using an unpaired *t*-test. A $P < 0.05$ was considered to be statistically significant.

Results

Psychophysics

CA self-desensitization

When 30 mM CA was reapplied bilaterally 5, 30, and 60 min following unilateral application of 30 mM CA, a significant majority of subjects chose the vehicle-pretreated side as having stronger irritation in the 2-AFC (Figure 1A) and assigned significantly higher intensity ratings to that side (Figure 1B). This is consistent with CA self-desensitization.

Menthol self-desensitization

When menthol (19 mM) was applied bilaterally either 5, 30, or 60 min following unilateral application of the same menthol concentration, a significant majority of subjects chose the vehicle-pretreated side to have greater irritation compared to the menthol-pretreated side in the 2-AFC (Figure 1C). In addition, subjects assigned significantly higher intensity ratings to the vehicle-treated versus menthol-pretreated side (Figure 1D). This indicates that menthol produced self-desensitization that persisted for 60 min or more.

CA cross-desensitization of menthol-evoked irritation

The lower concentration of CA (15 mM) did not cross-desensitize irritation elicited by subsequent application of a lower, intensity-matched concentration of menthol (19 mM) given 5 min later (i.e., no significant side preference in the 2-AFC, and no significant difference in bilateral intensity ratings). We therefore tested the effect of a higher concentration of CA (30 mM) on irritation elicited by a higher, intensity-matched concentration of menthol (29 mM). When menthol was applied bilaterally 5, 30, and 60 min after unilateral application of 30 mM CA, a significant majority of subjects chose the vehicle-pretreated side as having stronger irritation (Figure 2B) and assigned significantly higher ratings to that side (Figure 2A). This indicates that the higher concentration of CA elicited cross-desensitization that lasted 60 min or more.

Menthol cross-desensitization of CA-evoked irritation

We tested menthol cross-desensitization of CA-evoked irritation using a concentration of menthol (29 mM) that was matched in terms of the intensity of irritation elicited by 30 mM CA. After unilateral menthol, a significant majority of subjects chose the vehicle-pretreated side to have stronger irritation compared to the menthol-pretreated side (Figure 2C). However, subjects assigned significantly higher ratings to the vehicle-pretreated side when tested 5, but not 30 min, after unilateral menthol. This indicates a short-lasting cross-desensitizing effect of menthol on CA-evoked irritation. The discrepancy between the significant cross-desensitization after 30 min in the 2-AFC, but not bilateral intensity ratings, suggests that the 2-AFC is more sensitive than ratings to assess intensity differences between the 2 sides of the tongue.

Calcium imaging of TG cells

Of 1274 TG cells, 62% responded to menthol, CA, and/or capsaicin. The proportions of TG cells responsive to one or more chemicals are shown in Figure 3, where it can be seen that 49%, 17%, and 19% responded to capsaicin, menthol, and CA, respectively. That approximately half of menthol-sensitive TG cells responded to CA, and vice versa (Figure 3) is consistent with one recent study (Karashima et al. 2007) but not others (e.g., Kobayashi et al. 2005; Hjerling-Leffler et al. 2007).

CA self-desensitization

Figure 4A shows photomicrographs, and Figure 4B shows graphs of 3 TG cells' responses to repeated application of CA followed by menthol, capsaicin, and K^+. All cells exhibited some degree of tachyphylaxis to the second CA application at a 5-min interstimulus interval. Cell 1 but not 2 or 3 also responded to menthol, whereas cells 2 and 3, but not 1, responded to capsaicin. Overall, the mean peak baseline-corrected response to the second application of 200 μM CA application was not significantly smaller than the first, indicating an absence of self-desensitization or tachyphylaxis (Figure 5A). However, the second response to application of the higher (400 μM) CA concentration was significantly smaller compared to the first (Figure 5B). We interpret this to indicate concentration-dependent self-desensitization (tachyphylaxis).

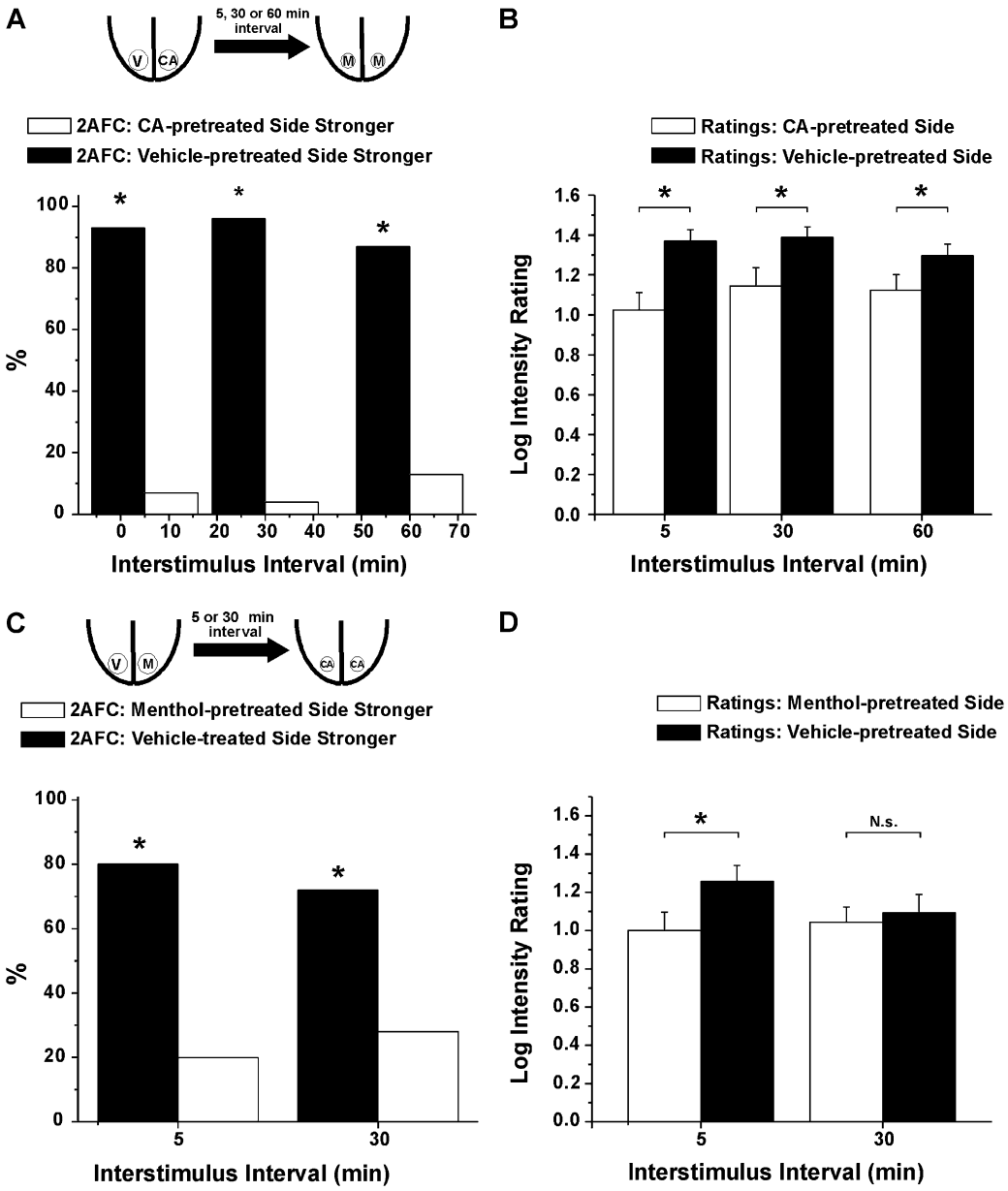

Figure 2 Cross-desensitization of CA- and menthol-evoked irritation. **(A)** 2-AFC: CA. At all interstimulus intervals, menthol (29 mM) evoked stronger irritation on the vehicle-pretreated versus CA-pretreated side in a significant proportion of subjects, indicating that CA (30 mM) cross-desensitized menthol irritation. **(B)** Intensity ratings. CA pretreatment significantly reduced irritation elicited by application of menthol 5, 30, and 60 min later. Lower CA concentration (15 mM) did not cross-desensitize menthol irritation. **(C)** 2-AFC: menthol. At both interstimulus intervals, CA (30 mM) evoked stronger irritation on the vehicle- versus menthol-pretreated side in a significant proportion of subjects, indicating that menthol (29 mM) cross-desensitized CA irritation. **(D)** Menthol significantly reduced irritation elicited by application of CA 5, but not 30, min later.

Menthol self-desensitization

Sequential application of menthol (250 μM) at a 5-min interstimulus interval resulted in significant self-desensitization (Figure 5C), indicating tachyphylaxis. However, when menthol was applied sequentially at a 10-min interstimulus interval, the second response was not significantly different compared to the first (Figure 5D). We interpret this to indicate that menthol induces self-desensitization (tachyphylaxis) at the shorter 5-min interval, but that this effect washes out within 10 min. By comparison, in vivo clearance of menthol from the lingual epithelium presumably takes considerably longer, thus allowing menthol to exert considerably longer self-desensitization in the psychophysical studies compared to the TG cells in vitro.

CA cross-desensitization of menthol-evoked responses

Using the first paradigm, we recorded TG cell responses to CA followed 5 min later by menthol. We compared the

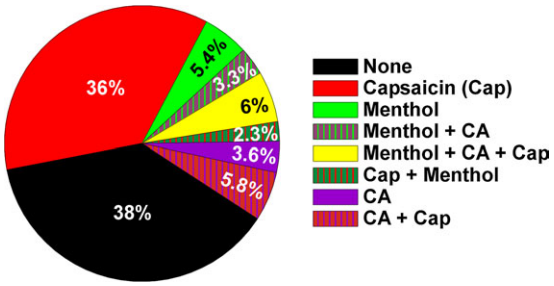

Figure 3 Proportions of TG cells responsive to menthol, CA, and/or capsaicin. Of 1274 K^+-responsive TG cells, 49% responded to capsaicin, 17% to menthol, and 19% to CA. Percentages of cells responsive to one or more chemicals are indicated within each sector. Men, Menthol; CA, Cinnamaldehyde; and Cap, capsaicin.

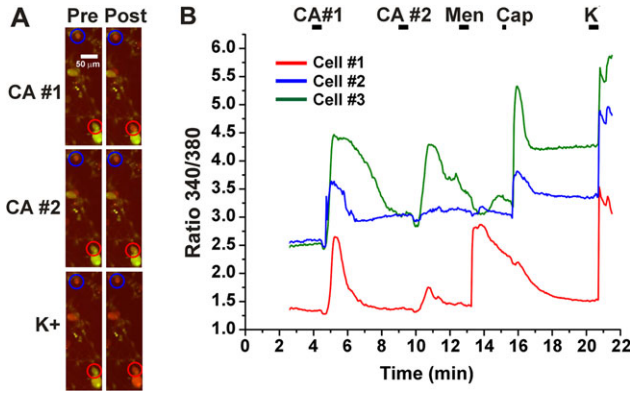

Figure 4 CA excitation of TG cells. **(A)** fluorescent images of 2 CA-sensitive TG cells (encircled). Each panel shows the 2 cells before (left-hand panel) and after (right panel) application of 400 μM CA (upper), a second application of CA (middle) and K^+ (lower). **(B)** Graph plots 340/380 nm ratio versus time for 3 TG cells including the 2 shown in A. All cells were activated by initial application of 400 μM CA and exhibited reduced responses to a second application of CA. Cell 1, but not 2 or 3, also responded to menthol, whereas cells 2 and 3, but not 1, responded to capsaicin. All cells responded to application of high-K^+ Ringers.

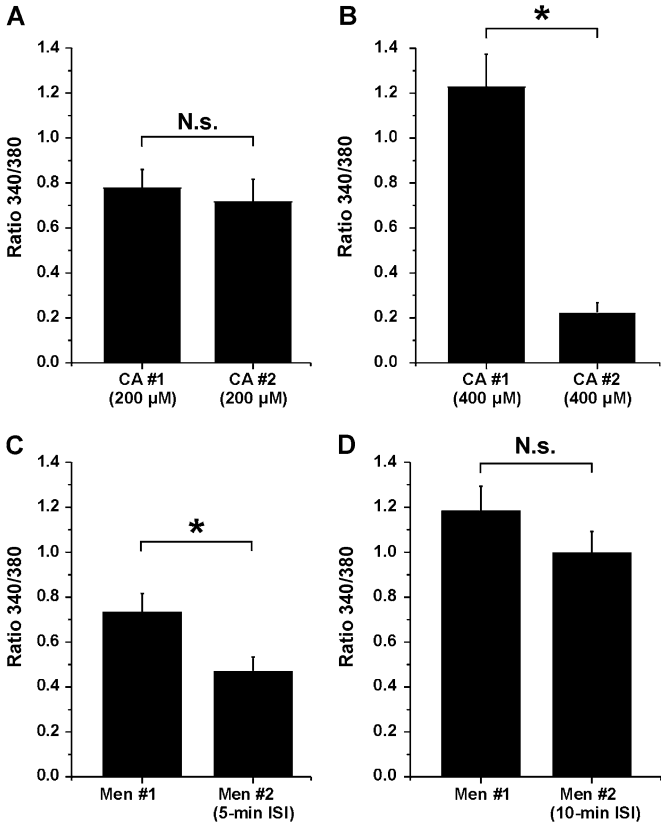

Figure 5 Self-desensitization (tachyphylaxis) of CA and menthol excitation of TG cells. **(A)** Bar graph plots mean peak baseline-corrected responses of TG cells to the first and second applications of CA (200 μM) at a 5-min interstimulus interval (ISI). Second response was not significantly different from first, indicating lack of self-desensitization ($n = 24$). **(B)** as in A with higher (400 μM) CA concentration. The second response to CA was significantly lower compared with the first ($^*P < 0.05$, paired t-test; $n = 33$), indicating self-desensitization. **(C)**. As in A for menthol (250 μM) applied at 5-min ISI. The second response to menthol was significantly lower compared with the first ($P < 0.05$, paired t-test, $n = 24$). **(D)** As in C with 10-min ISI between successive applications of menthol. Mean responses were not significantly different, indicating lack of self-desensitization ($n = 33$).

menthol response post-CA with responses of a separate population of cells to menthol when it was tested first. This analysis revealed that the mean response to menthol post-200μM CA was not significantly different compared with the initial menthol-evoked response, whereas the response to menthol post-400 μM CA was (Figure 6A). This indicates that the higher (400 μM) concentration of CA cross-desensitized responses of TG cells to menthol.

In the second paradigm, we tested TG cell responses to menthol, followed 4 min later by CA followed 1 min later by a second application of menthol. Approximately 50% of the menthol-sensitive TG cells also responded to CA, whereas the other half did not. For the latter population, the response to the second application of menthol, immediately following CA, was significantly lower than the response to the first menthol application (Supplementary Figure 1A). A similar result was obtained when the second menthol application was preceded by a higher 400 μM CA concentration (Supplementary Figure 1B). For the approximately 50% of cells that responded to both menthol and CA, the response to the second application of menthol was superimposed on the falling phase of the CA-evoked response. Although the second menthol-evoked response was significantly smaller, interpretation of this result is confounded by the possibility that the TG cells may have still been partially saturated with calcium. Overall, the data are consistent with CA cross-desensitization of menthol-evoked responses of TG cells.

Menthol cross-desensitization of CA-evoked responses

Using the first paradigm, the responses of TG cells to CA (200 μM) following menthol (250 μM) were significantly

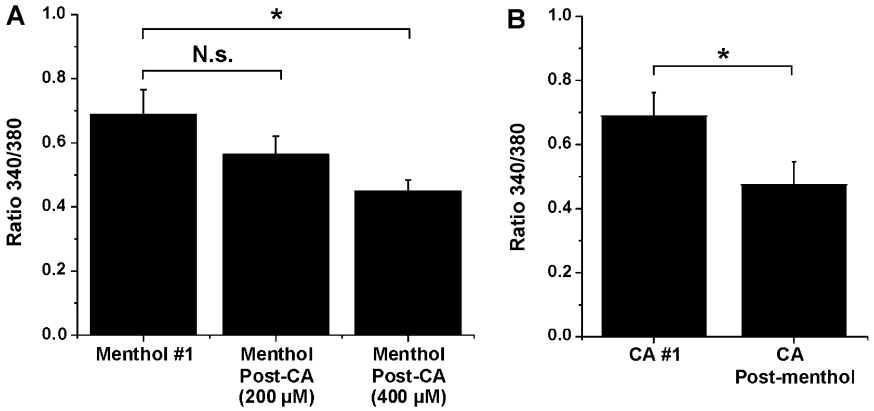

Figure 6 Cross-desensitization of menthol and CA excitation of TG cells. **(A)** Bar graph plots mean peak responses of TG cells to menthol (250 μM, first bar, $n = 35$) compared with menthol responses 5 min post exposure to CA at low (200 μM, middle bar, $n = 38$) or high concentration (400 μM, right bar, $n = 27$) ($^*P < 0.05$). **(B)** Mean peak responses of TG cells to CA (200 μM, $n = 40$) compared with CA responses 5 min post exposure to menthol (250 μM) ($^*P < 0.05$), $n = 34$.

lower compared with the initial response of other TG cells to menthol (Figure 6B). Because sequential application of 200 μM CA did not result in significant self-desensitization (Figure 5A), this result supports menthol cross-desensitization of CA-evoked responses. Using the second paradigm, a similar result was obtained for CA-sensitive and menthol-insensitive TG cells (Supplementary Figure 1C).

Discussion

The present psychophysical data confirm that menthol and CA elicit oral irritation that desensitizes across repeated applications and show for the first time that these chemicals induce reciprocal cross-desensitization of oral irritation. Similarly, TG cells responded to menthol and/or CA and also exhibited self-desensitization and mutual cross-desensitization of responses to CA and menthol at a short (5-min) interstimulus interval. These results indicate that the short-term temporal dynamics of oral irritancy, and interactions between CA and menthol, can be explained largely by the properties of peripheral trigeminal primary sensory neurons innervating the oral cavity. However, this does not preclude the possibility that menthol and CA activate primary afferents that can also interact centrally to modulate sensory transmission along the trigeminal pathway to affect perception.

Menthol, CA, and another TRPA1 agonist, AITC (allyl isothiocyanate; mustard oil), have been previously reported to elicit oral irritation in a temporally desensitizing pattern (Cliff and Green 1994, 1996; Prescott and Swain-Campbell 2000; Dessirier et al. 2001; Simons et al. 2003). In contrast, sequential application of capsaicin at 1-min interstimulus intervals elicited an increasing (sensitizing) pattern of irritation (Green 1989; Dessirier et al. 1997). Menthol, CA, and capsaicin all exhibited self-desensitization at interstimulus intervals >5 min (Green 1989; Simons et al. 2003). Presently, self-desensitization elicited by menthol and the higher CA concentration (30 mM) persisted at least 60 min. By comparison, self-desensitization of oral irritation elicited by capsaicin at high (>330 μM) concentrations can persist for more than 48 h (Karrer and Bartoshuk 1991; Carstens et al. 2007). From a practical viewpoint, this means that oral hygiene and other products containing menthol or CA can reduce subsequent oral chemesthetic sensitivity for some time after their use.

In rats, menthol applied by constant flow to the tongue excites cold-sensitive superficial Vc neurons with a subsequent progressive decline in firing (Zanotto et al. 2007), whereas constant or repetitive application of capsaicin elicited a progressive increase in Vc neuronal firing (Dessirier et al. 2000). For both chemicals, there was significant self-desensitization that persisted for at least 15 min. The self-desensitizing effects of menthol and CA may be mediated peripherally because TG cells exhibited reduced responses to repeated application of menthol and the higher concentration of CA at a 5-min interstimulus interval. However, menthol self-desensitization was lost at a longer, 10-min interstimulus interval, presumably due to rapid washout. This probably represents the biggest limitation in comparing the cellular and human psychophysical data. In the human study, topically applied menthol or CA must diffuse through lipid-rich tissue of the lingual epithelium to reach trigeminal nerve endings. Clearance of these agents from the lingual epithelium presumably takes considerable time, which likely explains the more prolonged self-desensitizing effect observed in the human psychophysical study compared with responses of TG cells recorded in vitro.

Menthol activates TRPM8 (McKemy et al. 2002; Peier et al. 2002; Bautista et al. 2007) and desensitizes it via a Ca^{++}-mediated depletion of phosphatidylinositol 4,5-bisphosphate (Rohacs et al. 2005) and/or protein kinase C-mediated phosphorylation (Abe et al. 2006). Desensitization of TRPM8 expressed in peripheral nerve endings of cold receptors or nociceptive afferents projecting to Vc neurons

therefore provides a reasonable explanation for the perceived reduction in oral irritation induced by sequential application of menthol. Because TRPA1 is expressed in nociceptive nerve endings (Story et al. 2003; Jordt et al. 2004), and menthol activates human TRPA1 (Xiao et al. 2008) and mouse TRPA1 at low concentrations (Karashima et al. 2007), then menthol activation of TRPA1 may also contribute to oral irritancy and induce self-desensitization via an as yet unknown mechanism.

We presently observed self-desensitization of oral irritation by 30 mM CA that persisted for at least 60 min. Repeated lingual application of CA (Prescott and Swain-Campbell 2000) and AITC (Simons et al. 2003) elicits a temporally desensitizing pattern of oral irritation with self-desensitization and cross-desensitization of capsaicin-evoked irritation. Similarly, AITC also excited rat Vc (Simons et al. 2004) and spinal dorsal horn neurons (Merrill et al. 2008) in a desensitizing temporal pattern. A higher (400 μM) but not lower (200 μM) concentration of CA resulted in self-desensitization of TG cell responses when applied at a 5-min interstimulus interval, consistent with the human psychophysical data, suggesting that the desensitizing action occurs peripherally. The mechanism underlying desensitization of TG cells to CA is not known. TRPA1 agonists such as CA covalently bind cysteine residues of TRPA1 (Macpherson et al. 2007) possibly preventing further activation. CA self-desensitization may also involve an intracellular calcium-mediated (Wang et al. 2008) or calcium-independent (Akopian et al. 2007) inactivation of TRPA1, and/or trafficking of TRPA1 to and from the cell membrane (Schmidt et al. 2009).

Menthol and CA were presently shown to exhibit a reciprocal cross-desensitization of oral irritation, consistent with our previous study showing reciprocal cross-desensitization of responses of rat Vc neurons to lingual application of menthol and CA (Zanotto et al. 2008). Menthol presently cross-desensitized TG cell responses to CA, possibly accounting for its cross-desensitization of CA-evoked oral irritation. Conversely, a high (30 mM) but not low (15 mM) CA concentration cross-desensitized oral irritation elicited by menthol. Similarly, a high (400 μM) but not low (200 μM) concentration of CA cross-desensitized TG cell responses to menthol. We previously reported that AITC exhibited self-desensitization and cross-desensitized oral irritation elicited by capsaicin (Simons et al. 2003), and CA cross-desensitized responses of rat Vc neurons to menthol (Zanotto et al. 2008), consistent with the present data. Menthol cross-desensitization of TG cell responses to CA might be attributed to menthol's reported ability to inhibit TRPA1 (Macpherson et al. 2006) particularly at menthol concentrations of 250 μM or higher (Karashima et al. 2007). Menthol also activates TRPA1 (see above), and it is conceivable that covalent binding of CA to cysteine residues of TRPA1 might prevent menthol from subsequently binding TRPA1, representing another mechanism for CA cross-desensitization by menthol. Another potential mechanism is the reported ability of CA to dose-dependently inhibit menthol activation of TRPM8 (Macpherson et al. 2006).

Supplementary material

Supplementary material can be found at http://www.chemse.oxfordjournals.org/.

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