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EUGENOL AND CARVACROL EXCITE FIRST- AND SECOND-ORDER TRIGEMINAL NEURONS AND ENHANCE THEIR HEAT-EVOKED RESPONSES

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Abstract—Eugenol and carvacrol from clove and oregano, respectively, are agonists of the warmth-sensitive transient receptor potential channel TRPV3 and the irritant-sensitive transient receptor potential ankyrin (TRPA)-1. Eugenol and carvacrol induce oral irritation that rapidly desensitizes, accompanied by brief enhancement of innocuous warmth and heat pain in humans. We presently investigated if eugenol and carvacrol activate nociceptive primary afferent and higher order trigeminal neurons and enhance their heat-evoked responses, using calcium imaging of cultured trigeminal ganglion (TG) and dorsal root ganglion (DRG) neurons, and *in vivo* single-unit recordings in trigeminal subnucleus caudalis (Vc) of rats. Eugenol and carvacrol activated 20–30% of TG and 7–20% of DRG cells, the majority of which additionally responded to menthol, mustard oil and/or capsaicin. TG cell responses to innocuous (39°) and noxious (42 °C) heating were enhanced by eugenol and carvacrol. We identified dorsomedial Vc neurons responsive to noxious heating of the tongue in pentobarbital-anesthetized rats. Eugenol and carvacrol dose-dependently elicited desensitizing responses in 55% and 73% of heat-sensitive units, respectively. Responses to noxious heat were briefly enhanced by eugenol and carvacrol. Many eugenol- and carvacrol-responsive units also responded to menthol, cinnamaldehyde and capsaicin. These data support a peripheral site for eugenol and carvacrol to enhance warmth- and noxious heat-evoked responses of trigeminal neurons, and are consistent with the observation that these agonists briefly enhance warmth and heat pain on the human tongue.

Key words: warmth, heat pain, rat, trigeminal ganglion cell, eugenol, carvacrol.

INTRODUCTION

Eugenol and carvacrol are organic chemicals found in clove and oregano, respectively. These compounds have antiseptic and flavor-additive properties, and are used in a variety of commercial applications. Eugenol has been used in dentistry as a local anesthetic (Markowitz et al., 1992) owing to its inhibitory effect on voltage-gated sodium and calcium channels in trigeminal nociceptors (Lee et al., 2005; Park et al., 2006, 2009; Chung et al., 2008). Carvacrol has also been reported to have anti-nociceptive effects (Cavalcante Melo et al., 2012). Additionally, eugenol and carvacrol elicit oral pungency (Cliff and Heymann, 1992; Klein et al., 2013) and eugenol activates transient receptor potential ankyrin (TRPA)-1 and transient receptor potential vanilloid (TRPV)-1 (Bandell et al., 2004) that are expressed in nociceptive nerve endings. Eugenol enhanced presynaptic glutamate release in the rat superficial spinal cord dorsal horn via an action at TRPA1 (Inoue et al., 2012). Carvacrol activates human and mouse TRPA1 (Bandell et al., 2004; Xu et al., 2006; Lee et al., 2008; de la Roche et al., 2013). A common feature of both compounds is that they activate TRPV3 (Xu et al., 2006; Vogt-Eisele et al., 2007; Sherkheli et al., 2009), which is expressed in sensory neurons and keratinocytes and is activated by innocuous warming (Peier et al., 2002; Smith et al., 2002; Xu et al., 2002; Chung et al., 2004). Previous reports suggested that TRPV3 also contributes to heat pain in mice (Moqrich et al., 2005; Huang et al., 2008), although this has been disputed since knockout mice lacking TRPV3 exhibited little or no change in thermal preference behavior or acute heat nociception (Huang et al., 2011).

In humans, eugenol and carvacrol elicited oral and nasal irritation consisting of warming, cooling, burning, stinging, pricking, tingling and numbing subqualities (Cliff and Heymann, 1992; Green, 2002; Wise et al., 2012; Klein et al., 2013) similar to those elicited by other TRP channel agonists (Dessirier et al., 2001; Simons et al., 2003; Albin et al., 2008; Bennett and Hayes, 2012). Moreover, both eugenol and carvacrol enhanced the perceived intensity of innocuous warmth as well as heat pain on the tongue (Klein et al., 2013). Collectively,

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Abbreviations: AITC, allyl isothiocyanate; ANOVA, analysis of variance; CA, cinnamaldehyde; Cap, capsaicin; FPP, farnesyl phosphopyruvate; PSTH, peristimulus time histograms; SEM, standard error of the mean; TRPA, transient receptor potential ankyrin; TRPV, transient receptor potential vanilloid; Vc, trigeminal subnucleus caudalis.

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these studies suggest that eugenol and carvacrol have both pro- and anti-nociceptive effects via their actions at TRPV3, TRPA1 and TRPV1 expressed in peripheral and central primary afferent terminals.

There are few previous studies of the ability of eugenol and carvacrol to directly excite primary sensory or higher order trigeminal neurons (Ohkubo and Kitamura, 1997). We presently investigated if these chemicals excite trigeminal ganglion (TG) and dorsal root ganglion (DRG) neurons, including those responsive to thermal stimuli, using the method of flourometric calcium imaging. Since many irritants activate neurons in trigeminal subnucleus caudalis (Vc; Carstens et al., 1998; Zanotto et al., 2007), we also used *in vivo* electrophysiological methods to investigate if eugenol and carvacrol activate Vc neurons and enhance their responses to warmth and/or noxious heat. An abstract of a portion of this work has appeared previously (Klein et al., 2012b).

EXPERIMENTAL PROCEDURES

All experiments were conducted under protocols approved by the UC Davis Institutional Animal Care and Use Committee.

Calcium imaging

Trigeminal ganglia (TG) and lumbrosacral dorsal root ganglia (DRG) were extracted from juvenile (2–3 weeks) male Sprague–Dawley rats ($n = 20$). The ganglia were triturated and TG and DRG cells were processed as previously described (Klein et al., 2011a,b) and plated onto glass coverslips pre-treated with poly-D-lysine. Cells were given fresh media after 1 h and imaged within 48 h.

After loading with 10 μM Fura-2AM (F1221, Invitrogen, Grand Island, NY, USA) in Ringer's solutions (140 mM NaCl, 4 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, 4.54 mL NaOH, and pH adjusted to 7.4), TG and DRG cells were placed in a perfusion chamber (CSC-25, Bioscience Tools, San Diego, CA, USA) on a thermal stage (BTC-S and BTC-100, Bioscience Tools) set at 34 °C. Images were taken every 3 s with NIS Elements software (Nikon Instruments Inc., Melville, NY, USA) at 340/380-nm wavelengths. Solutions were administered by a gravity-fed solenoid operated perfusion system (ValveLink 8.2, AutoMate Scientific, Berkeley, CA, USA) and removed via vacuum line at the other end. Chemicals used included 250 μM menthol (Givaudan Flavors Corporation, Cincinnati, OH, USA), 100 μM AITC (allyl isothiocyanate; mustard oil; Sigma–Aldrich Chemical Co., St. Louis, MO, USA), 200 μM eugenol (Sigma), 100 μM carvacrol (Sigma), and 1 μM capsaicin (Cap, Sigma). All chemicals were dissolved in 0.015% ethanol Ringer's solution. In separate experiments, TG and DRG cells were tested for sensitivity to 1 μM farnesyl phosphopyruvate (FPP, Enzo Life Sciences, Farmingdale, NY, USA) in 100 μM NH_4HCO_3 and 0.0028% ethanol. Chemicals were delivered for 30 s, with the exception of Cap which was delivered for 10 s. Ringer's solution containing a high $K+$ concentration (144 mM) was given at the end of the experiment to verify neuronal recordings. Vehicle (0.015% ethanol in Ringer's) did not have any effect (data not shown).

In a separate group of experiments we investigated thermal responses. TG and DRG cells were perfused with Ringer's solution pre-heated to either 39 or 42 °C for 30 s using a miniature heater (TC-RD, Bioscience Tools) connected to a separate temperature controller (BTC-1-100, Bioscience Tools). The bath temperature was monitored by a thermocouple within the feedback-controlled thermal stage and also by a separate thermocouple (IT-18, Physitemp Instruments, Inc., Clifton, NJ, USA) placed in the bath just outside the microscopic field of view. The thermocouple was connected to a microprobe thermometer (BAT-12, Physitemp) which was fed via a Powerlab interface (AD Instruments, Colorado Springs, CO, USA) to a digital computer and viewed using Chart 5 software. Following delivery of the first heat stimulus (either 39 or 42 °C), a second equivalent heat stimulus was delivered 10 min later. The second heat stimulus was preceded 30 s earlier by bath delivery of either eugenol, carvacrol or no stimulus.

Single unit recording

Methods were essentially the same as described previously (Zanotto et al., 2007, 2008; Klein et al., 2011c). Eighty-three adult male Sprague–Dawley rats (480 ± 8.3 g) were anesthetized with sodium pentobarbital (induction: 65 mg/kg i.p., maintenance: 10 mg/kg i.v.). The caudal medulla was exposed surgically while body temperature was maintained by a heating pad and oxygen delivered via tracheal cannula. The electrocardiography (ECG) was recorded and displayed continuously using a Powerlab interface (AD Instruments). A tungsten microelectrode (FHC, Bowdoin, ME, USA; 10 MΩ) was positioned using a hydraulic microdrive (David Kopf Instruments, Tujunga, CA, USA) to record single Vc units having heat-sensitive lingual receptive fields. We used a noxious heat stimulus (see below) to isolate Vc units and did not attempt to identify units responsive to innocuous warming that are quite rare (Dostrovsky and Hellon, 1978; Andrew and Craig, 2001). Unit activity was amplified and displayed using Powerlab and Spike 2 (Cambridge Electronic Design, Cambridge, UK) interfaces. In some experiments more than one heat sensitive unit was recorded and discriminated by waveform post hoc using the Spike 2 software. Only units that responded to noxious heat were selected for further analysis.

Thermal stimuli were delivered using a feedback-controlled Peltier thermode (NTE-2A, Physitemp, Clifton, NJ, USA; 13 mm diameter) attached to a micromanipulator to contact the dorsal anterior surface of the tongue. The Peltier thermode was programed to deliver noxious heat (up to 53 °C) followed 2 min later by cooling (down to 7 °C) from an adapting temperature of 35 °C (Klein et al., 2012b). The lingual–thermode interface temperature was measured using a fast thermocouple (IT-18, Physitemp) connected to a microprobe thermometer (BAT-12, Physitemp) and was displayed continuously using a Powerlab interface and Chart software (AD Instruments). The heat–cold sequence was delivered twice and