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Original article

Moieties of plant-derived compounds responsible for outward current production and TRPA1 activation in rat spinal substantia gelatinosa

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ABSTRACT

Background: Transient receptor potential ankyrin-1 (TRPA1) channels expressed in the central terminal of dorsal root ganglion neurons in the spinal substantia gelatinosa (SG) play a role in modulating nociceptive transmission. Although plant-derived compounds exhibiting antinociception (such as eugenol, carvacrol and thymol) activate TRPA1 channels to enhance spontaneous excitatory transmission while hyperpolarizing membranes in SG neurons without TRPA1 activation, specific chemical moieties involved in synaptic modulation are unknown.

Methods: We examined the effects of other plant-derived compounds (guaiacol, vanillin, vanillic acid and *p*-cymene) on holding current and spontaneous excitatory transmission at −70 mV by applying the whole-cell patch-clamp technique to SG neurons in adult rat spinal cord slices.

Results: None of the compounds affected the frequency or amplitude of spontaneous excitatory postsynaptic current. Guaiacol and vanillic acid had no effect on holding currents, while vanillin and *p*-cymene produced an inward and outward current, respectively, in some neurons tested. Synaptic modulation was also observed within the same neuron as the activities of eugenol, carvacrol, thymol, and the chemically-related plant-derived compound zingerone occurred.

Conclusion: A substituted group in eugenol and zingerone, but not in guaiacol, vanillin or vanillic acid, as well as an —OH bound to the benzene ring of carvacrol and thymol, but not *p*-cymene, play a role in producing outward current and TRPA1 activation. Thus, the binding of such chemical moeties to the benzene ring of plant-derived compounds appears necessary to modulate nociceptive transmission in the SG. This information provides insight for the development of new analgesics based on plant-derived compounds.

Introduction

Information about nociceptive stimuli detected in the periphery is transferred through dorsal root ganglion (DRG) neurons to lamina II (substantia gelatinosa; SG) neurons of the spinal dorsal horn, which play a crucial role in regulating nociceptive transmission [1]. Transmission of this information is inhibited in SG neurons by various endogenous analgesics, such as opioids, noradrenaline and adenosine. Membrane hyperpolarization is among the cellular mechanisms underlying this inhibition [2].

Transient receptor potential (TRP) ankylin-1 (TRPA1) and TRP vanilloid-1 (TRPV1) channels expressed in the central terminal of DRG neurons in the SG are involved in modulation of nociceptive transmission [3]. This concept is supported by observations that peripheral inflammation results in excessive expression of TRPA1 channels in the mouse spinal cord and DRG; moreover, intrathecal administration of TRPA1 antagonist reverses hyperalgesia in mouse models of neuropathic pain [4]. In *in vitro* spinal cord slice preparations, central terminal TRPA1 channels are activated by various plant-derived chemicals, resulting in enhanced spontaneous release of L-glutamate from nerve terminals onto SG neurons, which in turn increases their membrane excitability [3,5].

We previously found that eugenol, carvacrol and thymol, all of which are plant-derived, activate TRPA1 but not TRPV1 channels to result in enhanced spontaneous release of L-glutamate. Moreover, these plant-derived chemicals produced an outward current at a holding potential (V_H) of −70 mV, *i.e.*, membrane hyperpolarization, in adult rat SG neurons in a manner independent of TRPA1 and TRPV1 activation [6–8]. Such hyperpolarization could counteract the increased membrane excitability produced by TRPA1 activation. Other plant-derived chemicals, including allyl isothiocyanate,

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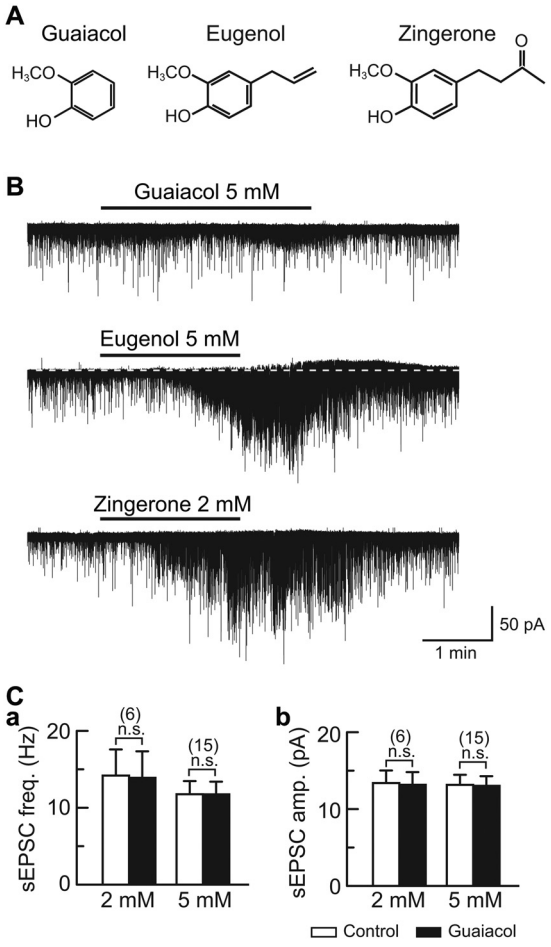

Fig. 1. Guaiacol neither produces an outward current nor enhances glutamatergic spontaneous excitatory transmission in substantia gelatinosa (SG) neurons, in contrast to eugenol or zingerone. (A) Chemical structures of guaiacol, eugenol and zingerone. (B) Chart recordings showing changes in holding current and spontaneous excitatory transmission following superfusion of 5 mM guaiacol (top), 5 mM eugenol (middle) or 2 mM zingerone (bottom); recordings were obtained from the same neuron. Note that eugenol and zingerone, but not guaiacol, enhanced spontaneous excitatory transmission, while only eugenol produced an outward current. (C) Average spontaneous excitatory postsynaptic current (sEPSC) frequency (a) and amplitude (b), which were measured for 30 s, before (Control) and approximately 3 min after addition of 2 mM or 5 mM guaiacol (Guaiacol). In this and subsequent figures, duration of drug superfusion is indicated by a horizontal bar above the chart recording; holding current level in the control is indicated by a dotted line; values in parentheses indicate numbers of neurons tested; each point with vertical bars represents mean and SEM; n.s.: not significant. Holding current (V_H) – −70 mV.

cinnamalaldehyde, allicin, zingerone, 1,8-cineole, (+)-carvone and citral, activated central terminal TRPA1, but not TRPV1, channels while producing no or only a small inward current, and no outward current in SG neurons [9–13]. The outward current produced by carvacrol has been shown to arise from activation of a K^+ channel in SG neurons [7].

Eugenol-, carvacrol- and thymol-induced membrane hyperpolarization in SG neurons may contribute to antinociception produced by their intrathecal or oral administration [14–16], as observed for endogenous analgesics. To elucidate which chemical moieties of these compounds are involved in producing outward current and TRPA1 activation, we examined effects of plant-derived chemicals guaiacol, vanillin, vanillic acid and *p*-cymene, which are similar in chemical structure to eugenol, carvacrol and thymol, on holding current and spontaneous excitatory transmission at −70 mV by applying the whole-cell patch-clamp technique to SG neurons in adult rat spinal cord slices. In addition, we investigated the action of zingerone, another plant-derived compound with the ability to activate TRPA1 channels in the SG [10], because its chemical structure is similar to guaiacol, vanillin and vanillic acid.

Materials and methods

Slice preparation

All animal experiments were approved by the Animal Care and Use Committee of Saga University. Slice preparations from adult rat spinal cords were prepared as described elsewhere [12,13]. Briefly, adult male Sprague-Dawley rats (6–8 weeks old; 200–300 g) were anesthetized with urethane (1.5 g/kg body weight, *ip*) and then the lumbosacral segment (L_1-S_3) of the spinal cord was extracted and placed in cold pre-oxygenated Krebs solution (2–4 °C) pre-equilibrated with 95% O_2 and 5% CO_2. After cutting all ventral and dorsal roots, the pia-arachnoid membrane was removed. The spinal cord was mounted on a microslicer (DTK-1000, Dousaka, Kyoto, Japan) and then a 650 μm-thick transverse slice was cut. The slice was transferred to a recording chamber (volume = 0.5 mL), completely submerged and then superfused at a rate of 12–15 mL/min with Krebs solution saturated with 95% O_2 and 5% CO_2 at 36 ± 1 °C. The composition of Krebs solution was (in mM): 117 NaCl, 3.6 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 1.2 NaH_2PO_4, 25 $NaHCO_3$ and 11 glucose.

Whole-cell patch-clamp recordings

Blind whole-cell voltage-clamp recordings from SG neurons were made at a V_H of −70 mV with a patch-pipette, as previously described [12,13]. The patch-pipette solution was composed of (in mM): 135 K-gluconate, 0.5 $CaCl_2$, 2 $MgCl_2$, 5 KCl, 5 EGTA, 5 HEPES and 5 Mg-ATP. Signals were acquired using an amplifier (Axopatch 200B; Molecular Devices, Sunnyvale, CA, USA). Currents obtained in voltage-clamp mode were low-pass-filtered at 3 kHz and digitized at 333 kHz with an A/D converter (Digidata 1322 A, Molecular Devices). Data were stored and analyzed with a personal computer. Spontaneous excitatory postsynaptic currents (sEPSCs) were detected and analyzed using Mini Analysis Program (ver. 6.0.3; Synaptosoft, Decatur, GA, USA); when sEPSC frequency and amplitude changed >5% following superfusion of a drug, the effect of this drug on excitatory transmission was considered to be effective, as previously described [12,13]. Numerical data are given as mean ± SEM. Statistical significance was determined as $p < 0.05$ using the paired Student's *t*-test. In all cases, *n* refers to the number of neurons studied.

Application of drugs

Drugs were applied by perfusing a solution containing drugs at known concentrations without changing the perfusion rate or temperature. Drugs used included guaiacol, *p*-cymene (Tokyo Chemical Industries, Co. Ltd., Tokyo, Japan), vanillin and vanillic acid (Sigma-Aldrich, St Louis, MO, USA), which were first dissolved in dimethyl sulfoxide (DMSO) at >100 times the concentration to be used and then stored at −20 °C. The stock solution was diluted to the desired concentration in Krebs solution immediately before use. DMSO (1%), which was used as a vehicle to dissolve drugs, had no effect on sEPSC frequency or amplitude, or holding currents [12].

Results

Guaiacol action

First, we examined the effect of guaiacol [2-methoxyphenol; lacks $—CH_2CH{=}CH_2$ or $—(CH_2)_2COCH_3$ bound to the benzene ring

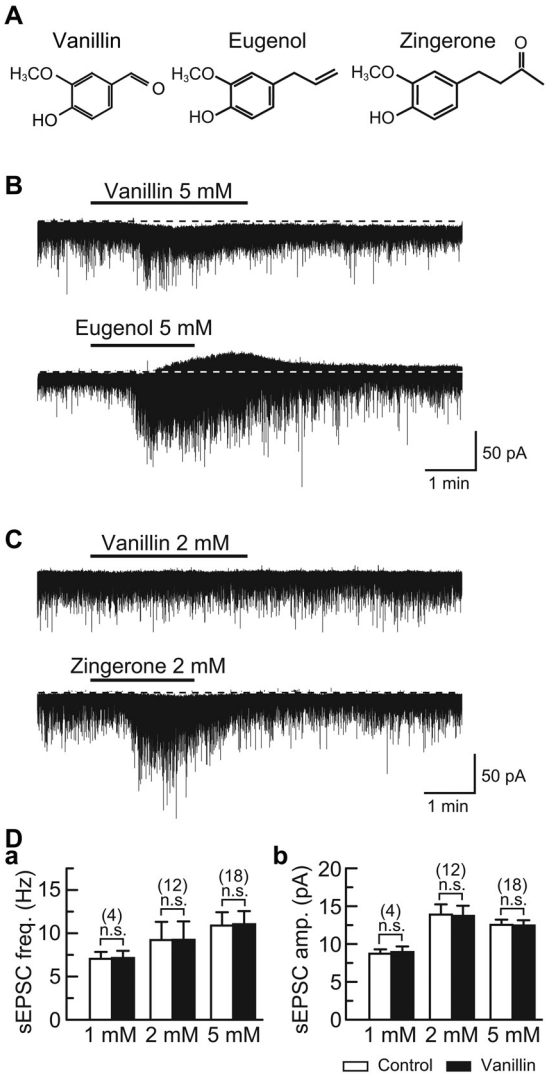

Fig. 2. Vanillin neither produces an outward current nor enhances spontaneous excitatory transmission in SG neurons, in contrast to eugenol or zingerone. (A) Chemical structure of vanillin; for comparison, structures of eugenol and zingerone are also shown. (B, C) Chart recordings showing changes in holding current and spontaneous excitatory transmission following superfusion of 5 mM vanillin or eugenol (B), or 2 mM vanillin or zingerone (C). In (B) and (C), top and bottom recordings were obtained from the same neuron. Note that eugenol and zingerone, but not vanillin, enhanced spontaneous excitatory transmission. Moreover, 5 mM vanillin and eugenol produced an inward and outward current, respectively. (D) Average sEPSC frequency (a) and amplitude (b), which were measured for 30 s, before (Control) and approximately 3 min after addition of 1, 2 or 5 mM vanillin (Vanillin). V_H –` –70 mV.

of eugenol or zingerone (Fig. 1A); contained in beechwood; [17]] on spontaneous excitatory transmission in SG neurons. Unlike eugenol, superfusion of 5 mM guaiacol for 3 min did not change holding currents ($n = 15$; for example, see Fig. 1B, top). Guaiacol at a lower concentration, such as 2 mM, also had no effect on holding currents ($n = 6$). Fig. 1C(a, b) shows average sEPSC frequency and amplitude values, which were measured for 30 s, before and approximately 3 min after the addition of 2 mM or 5 mM guaiacol. sEPSC frequency and amplitude were unaffected by guaiacol. Thus, the activity of guaiacol actions was different from that of eugenol and zingerone [6,10].

A lack of effect of 5 mM guaiacol was also observed in SG neurons sensitive to eugenol (5 mM), as shown in Fig. 1B, top and middle. Eugenol superfused for 2 min increased sEPSC frequency to 513% ± 82% ($p < 0.05$; $n = 5$) of levels observed prior to application (control). In addition, a small increase in sEPSC amplitude [139% ± 14% ($p < 0.05$) of control] around 2.5 min after the onset of superfusion and an outward current with average peak amplitude of 13.3 ± 1.8 pA ($n = 5$) were observed. In contrast, guaiacol changed neither sEPSC frequency nor amplitude [99% ± 2% ($p > 0.05$) and 99% ± 1% ($p > 0.05$; $n = 5$), respectively, of control], nor holding currents ($n = 5$).

Alternatively, SG neurons sensitive to zingerone (2 mM) did not respond to guaiacol (5 mM), as shown in Fig. 1B, top and bottom. Zingerone superfused for 2 min increased sEPSC frequency to 280% ± 43% ($p < 0.05$; $n = 6$) of control with a small increase in sEPSC amplitude [142% ± 15% ($p < 0.05$) of control] around 2.5 min after the onset of its superfusion. In 83% of neurons tested, this frequency increase was accompanied by an inward current with peak amplitude of 11.8 ± 1.4 pA ($n = 5$); the one remaining neuron did not change holding currents (Fig. 1B, bottom). In contrast, guaiacol changed neither sEPSC frequency nor amplitude [99% ± 1% ($p > 0.05$) and 99% ± 2% ($p > 0.05$; $n = 6$), respectively, of control], nor holding currents ($n = 6$).

Vanillin action

Next, we examined the effect of vanillin [4-hydroxy-3-methoxybenzaldehyde; —$CH_2CH{=}CH_2$ or —$(CH_2)_2COCH_3$ bound to the benzene ring of eugenol or zingerone is replaced by —CHO (Fig. 2A); contained in vanilla [18]] on spontaneous excitatory transmission in SG neurons. Vanillin at 1 mM did not change holding currents ($n = 4$). However, when examined at 2 mM, 25% of neurons examined ($n = 12$) had an inward current with peak amplitude of 9.0 ± 2.5 pA ($n = 3$), while other neurons examined did not (for example, see Fig. 2C, top). As shown in the top of Fig. 2B, 5 mM vanillin produced an inward current in all neurons tested; this peak amplitude averaged to be 11.8 ± 1.4 pA ($n = 18$). In contarst, sEPSC frequency and amplitude were unaffected by 5 mM vanillin (for example, see Fig. 2B, top). Fig. 2D(a, b) shows average sEPSC frequency and amplitude values, which were measured for 30 s, before and approximately 3 min after the addition of 1, 2 or 5 mM vanillin. sEPSC frequency and amplitude were unaffected by vanillin. Thus, observed actions of vanillin were different from those of eugenol and zingerone [6,10].

In SG neurons, where vanillin (5 mM) produced an inward current with no effect on spontaneous excitatory transmission, the application of eugenol (5 mM) enhanced transmission and produced an outward current, as shown in Fig. 2B, top and bottom. Superfusion of eugenol for 2 min increased sEPSC frequency to 464% ± 39% ($p < 0.05$; $n = 7$) of control with a small increase in sEPSC amplitude [137% ± 14% ($p < 0.05$) of control] around 2.5 min after the onset of its superfusion. With respect to holding currents, as shown in the bottom of Fig. 2B, 71% of neurons tested ($n = 7$) exhibited a eugenol-induced outward current with average peak amplitude of 15.3 ± 2.1 pA ($n = 5$). The remaining two neurons did not change holding currents. In contrast, vanillin did not change sEPSC frequency or amplitude [104% ± 2% ($p > 0.05$) and 99% ± 1% ($p > 0.05$; $n = 7$), respectively, of control] and produced an inward current with average peak amplitude of 9.4 ± 1.3 pA ($n = 7$).

Alternatively, SG neurons sensitive to zingerone (2 mM) responded to vanillin (2 mM) in a manner different from zingerone, as shown in Fig. 2C, top and bottom. Superfusion of zingerone for 2 min increased sEPSC frequency to 322% ± 18% ($p < 0.05$; $n = 8$) of control with a small increase in sEPSC amplitude [131% ± 9% ($p < 0.05$) of control] around 2.5 min after the onset of its superfusion. With respect to holding currents, 88% of neurons tested ($n = 8$) exhibited a zingerone-induced inward current with average peak amplitude of 12.8 ± 2.6 pA ($n = 7$; for example, see Fig. 2C, bottom); the one remaining neuron did not change holding

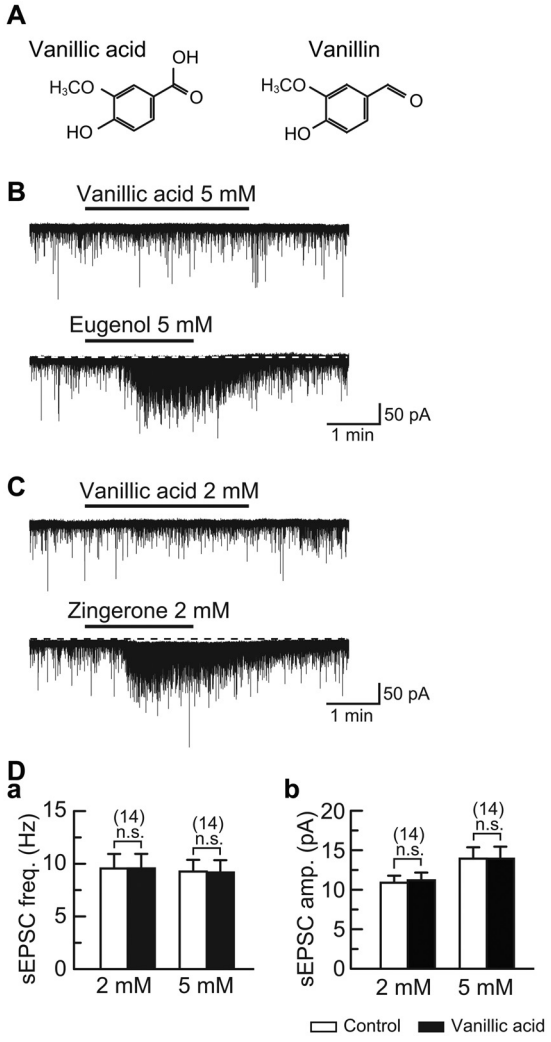

Fig. 3. Vanillic acid neither produces an outward current nor enhances spontaneous excitatory transmission in SG neurons, in contrast to eugenol or zingerone. (A) Chemical structure of vanillic acid; for comparison, the structure of vanillin is also shown. (B, C) Chart recordings showing changes in holding current and spontaneous excitatory transmission following superfusion of 5 mM vanillic acid or eugenol (B), or 2 mM vanillic acid or zingerone (C). In (B) and (C), top and bottom recordings were obtained from the same neuron. Note that eugenol and zingerone, but not vanillic acid, enhanced spontaneous excitatory transmission, whereas only eugenol produced an outward current. (D) Average sEPSC frequency (a) and amplitude (b), which were measured for 30 s, before (Control) and approximately 3 min after the addition of 2 mM or 5 mM vanillic acid (Vanillic acid). $V_H = -70$ mV.

currents. In contrast, vanillin changed neither sEPSC frequency nor amplitude [100% ± 1% ($p > 0.05$) and 99% ± 1% ($p > 0.05$; $n = 8$), respectively, of control] in all neurons tested, nor holding currents in seven out of eight neurons examined (for instance, see Fig. 2C, top). The one remaining neuron exhibited an inward current with peak amplitude of 5.3 pA.

Vanillic acid action

Exposure to a compound similar to vanillin, vanillic acid (4-hydroxy-3-methoxybenzoic acid; Fig. 3A; —CHO of vanillin is replaced by —COOH; contained in *Angelicae Radix*; [19]) at 2 mM ($n = 14$) and 5 mM ($n = 14$) had no effect on holding currents (see Fig. 3B and C, top). Fig. 3D(a, b) shows average sEPSC frequency and amplitude values, which were measured for 30 s, before and approximately 3 min after the addition of 2 mM or 5 mM vanillic acid. sEPSC frequency and amplitude were unaffected by vanillic acid. Thus, the actions of vanillic acid were different from those of eugenol and zingerone [6,10].

In SG neurons sensitive to eugenol (5 mM), vanillic acid (5 mM) had no effect on either holding currents or spontaneous excitatory transmission, as shown in Fig. 3B, top and bottom. Superfusion of eugenol for 2 min increased sEPSC frequency to 470% ± 55% ($p < 0.05$; $n = 5$) of control with a small increase in sEPSC amplitude [138% ± 11% ($p < 0.05$) of control] around 2.5 min after the onset of superfusion, and produced an outward current with average peak amplitude of 12.3 ± 2.2 pA ($n = 5$). In contrast, vanillic acid changed neither sEPSC frequency nor amplitude [98% ± 2% ($p > 0.05$) and 100% ± 2% ($p > 0.05$; $n = 5$), respectively, of control], nor holding currents ($n = 5$).

Alternatively, SG neurons sensitive to zingerone (2 mM) did not respond to vanillic acid (2 mM), as shown in Fig. 3C, top and bottom. Superfusion of zingerone for 2 min increased sEPSC frequency to 294% ± 31% ($p < 0.05$; $n = 8$) of control with a small increase in sEPSC amplitude [128% ± 11% ($p < 0.05$) of control] around 2.5 min after the onset of its superfusion. With respect to holding currents, 88% of neurons tested ($n = 8$) exhibited a zingerone-induced inward current with average peak amplitude of 9.6 ± 0.9 pA ($n = 7$; for example, see Fig. 3C, bottom). The one remaining neuron did not change holding currents. In contrast, vanillic acid changed neither sEPSC frequency nor amplitude [99% ± 2% ($p > 0.05$) and 102% ± 1% ($p > 0.05$; $n = 8$), respectively, of control], nor holding currents ($n = 8$).

p-Cymene action

Finally, we examined the effect of *p*-cymene [lacks —OH of carvacrol or thymol (Fig. 4A); contained in *Cymbopogon winterianus* Jowitt; [20]] on spontaneous excitatory transmission in SG neurons. As shown in Fig. 4B, superfusing *p*-cymene (2 mM) for 3 min produced an outward current in 15% of neurons tested ($n = 33$); this peak amplitude averaged to be 6.8 ± 2.8 pA ($n = 5$). Remaining neurons ($n = 28$) did not change holding currents (for example, see Fig. 4C, top). When exposed to 1 mM *p*-cymene ($n = 6$), two neurons exhibited outward currents with peak amplitudes of 6.4 and 21.6 pA, while the other neurons did not alter holding currents ($n = 4$). Fig. 4D(a, b) shows average sEPSC frequency and amplitude values, which were measured for 30 s, before and approximately 3 min after the addition of 1 mM or 2 mM *p*-cymene. sEPSC frequency and amplitude were unaffected by *p*-cymene. Thus, the actions of *p*-cymene were different from those of carvacrol and thymol [7,8].

A lack of effect of *p*-cymene (2 mM) was observed in SG neurons sensitive to carvacrol (1 mM), as shown in Fig. 4C, top and middle. In 63% of neurons tested ($n = 8$), superfusion of carvacrol for 2 min increased sEPSC frequency to 286% ± 19% ($p < 0.05$; $n = 5$) of control with a small increase in sEPSC amplitude [113% ± 8% ($p > 0.05$) of control] around 3.5 min after the onset of its superfusion, and produced an outward current with average peak amplitude of 13.7 ± 1.8 pA ($n = 5$; for instance, see Fig. 4C, middle). Two of the remaining three neurons exhibited an outward current (21.2 and 6.9 pA) without a change in sEPSC frequency (102% and 97%) or amplitude (99% and 97%), and one neuron increased sEPSC frequency and amplitude (224% and 114%, respectively, of control) without changing holding currents. In these neurons, *p*-cymene changed neither sEPSC frequency nor amplitude [102% ± 2% ($p > 0.05$) and 100% ± 2% ($p > 0.05$; $n = 8$), respectively, of control], nor holding currents ($n = 8$).

Alternatively, SG neurons sensitive to thymol (1 mM) did not respond to *p*-cymene (2 mM), as shown in Fig. 4C, top and bottom. Superfuion of thymol for 3 min increased sEPSC frequency to 375% ± 21% ($p < 0.05$; $n = 5$) of control with a small increase in sEPSC amplitude [129% ± 6% ($p < 0.05$) of control] around 5 min after the

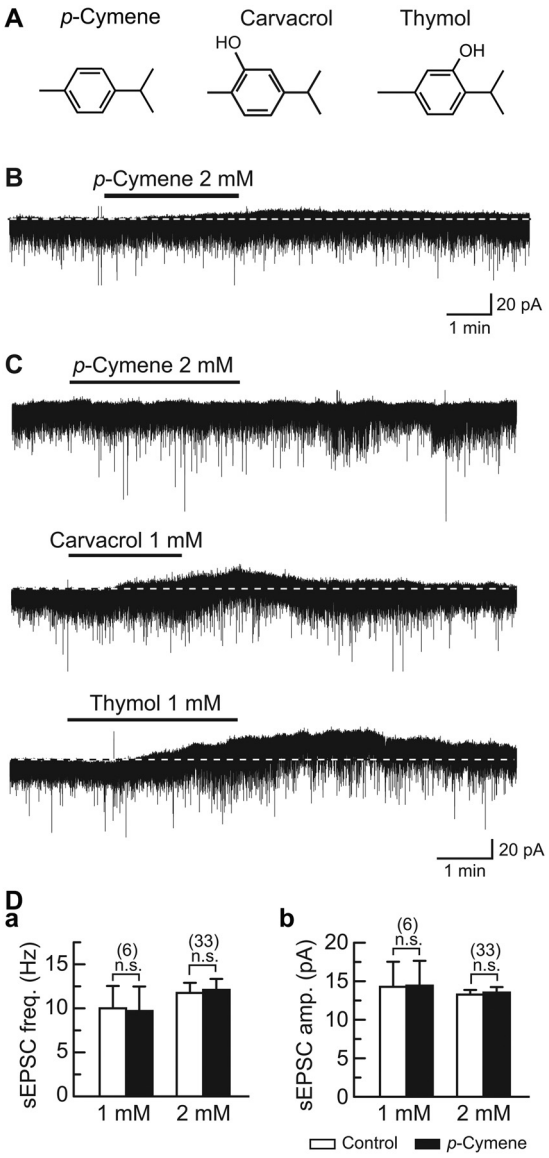

Fig. 4. *p*-Cymene affects holding currents without changing spontaneous excitatory transmission in SG neurons in a manner different from carvacrol and thymol. (A) Chemical structures of *p*-cymene, carvacrol and thymol. (B) Chart recording showing a small outward current following superfusion of *p*-cymene (2 mM). (C) Chart recordings showing changes in holding current and spontaneous excitatory transmission following superfusion of *p*-cymene (2 mM; top), carvacrol (1 mM; middle) and thymol (1 mM; bottom); recordings were obtained from the same neuron. Note that both carvacrol and thymol enhanced spontaneous excitatory transmission while producing an outward current. (D) Average sEPSC frequency (a) and amplitude (b), which were measured for 30 s, before (Control) and approximately 3 min after addition of 1 mM or 2 mM *p*-cymene (*p*-Cymene). V_H – −70 mV.

onset of its superfusion, and produced an outward current with average peak amplitude of 13.3 ± 2.6 pA ($n = 5$). However, *p*-cymene changed neither sEPSC frequency nor amplitude [102% ± 3% ($p > 0.05$) and 100% ± 2% ($p > 0.05$; $n = 5$), respectively, of control], nor holding currents ($n = 5$).

Discussion

The present study demonstrated that *p*-cymene produced an outward current at −70 mV (membrane hyperpolarization) in 15% of adult rat SG neurons tested, while guaiacol, vanillin and vanillic acid did not produce any outward currents. These observations differ from those of eugenol, carvacrol and thymol, which produced outward currents [6–8]. Unlike many plant-derived TRPA1 agonists, none of the compounds affected spontaneous excitatory transmission (sEPSC frequency and amplitude), indicating no activation of central terminal TRPA1 channels in the SG. This occurred even though their chemical structures are similar to eugenol, zingerone, carvacrol and thymol, all of which activate central terminal TRPA1 channels to result in increased sEPSC frequency in SG neurons [6–8,10]. Differences in synaptic modulation between guaiacol and eugenol (or zingerone); vanillin and eugenol (or zingerone); vanillic acid and eugenol (or zingerone); or *p*-cymene and carvacrol (or thymol) were confirmed within the same neuron. Vanillin and zingerone both produced a small inward current. Table 1 summarizes results obtained in the present and previous studies.

Moieties of plant-derived compounds involved in producing outward current and TRPA1 activation in the SG

As eugenol, but not guaiacol, produced an outward current in SG neurons, the —$CH_2CH{=}CH_2$ bound to the benzene ring of eugenol, but not guaiacol, is necessary for outward current production (see Fig. 1A). An outward current was not induced by either vanillin or vanillic acid, which have —CHO or —COOH, respectively, in place of —$CH_2CH{=}CH_2$ (see Fig. 3A). Although the —$CH_2CH{=}CH_2$ of eugenol does not have any electric charges, the —CHO of vanillin has a negative charge that is biased to the oxygen atom in the carbon-oxygen bond because of a difference in electronegativity between carbon and oxygen atoms; thus, this compound may not be able to produce outward currents. Consistent with this idea, zingerone, which has a —$(CH_2)_2COCH_3$ in place of the —CHO of vanillin [10], did not produce any outward current in SG neurons. Such prevention is also observed for the action of vanillic acid, whose —COOH dissociates to —COO^- and H^+ in Krebs solution, resulting in the existence of a negative charge in vanillic acid. This issue remains to be further examined by testing additional vanilloid compounds.

Although *p*-cymene is different from carvacrol or thymol only in terms of an —OH bound to the benzene ring (see Fig. 4A), *p*-cymene produced an outward current in just 15% of neurons examined. Moreover, the peak amplitude (6.8 pA at 2 mM) of this outward current was smaller than that observed for carvacrol (30.2 pA at 1 mM; [7]) and thymol (15.6 pA at 1 mM; [8]). This result suggests that the —OH in carvacrol and thymol plays a role in producing outward currents.

In the present study, we focused on the outward current produced by plant-derived chemicals, but it may be of interest to note that neither guaiacol, vanillin, vanillic acid nor *p*-cymene enhanced spontaneous excitatory transmission in rat SG neurons, in contrast to plant-derived agonists of not only TRPA1, but also TRPV1 and TRP melastatin-8 channels (see [5]). Indeed, addition of —$CH_2CH = CH_2$ (eugenol) or —$(CH_2)_2COCH_3$ (zingerone) to guaiacol would be necessary for TRPA1 activation in the SG (Fig. 1A). Moreover, replacement of the substituted groups with —CHO (vanillin) or —COOH (vanillic acid) (Fig. 3A), or deletion of —OH from carvacrol or thymol (*p*-cymene; Fig. 4A), resulted in a lack of TRPA1 activation. Hinman et al. [21] reported that activation of TRPA1 channels by various compounds is mediated by covalent modification of cysteine residues within the cytoplasmic N terminus of the channel. This may result from less electrophilicity of modifications for the actions of guaiacol, vanillin and vanillic acid, compared with eugenol and zingerone, and for the action of *p*-cymene, compared with carvacrol and thymol. Differences in TRPA1 activation remain to be further examined by testing additional related plant-derived compounds. Importantly, as

Table 1
Changes in sEPSC frequency and holding currents in rat SG neurons recorded at V_H of −70 mV following superfusion of plant-derived compounds.[*1]

Plant-derived compound	sEPSC frequency increase	Outward current	Inward current
Guaiacol	–	–	–
Vanillin	–	–	+
Vanillic acid	–	–	–
p-Cymene	–	+	–
Eugenol	+[*2]	+[*3]	–
Zingerone	+[*2]	–	+
Carvacrol	+[*2]	+[*3]	–
Thymol	+[*2]	+[*3]	–

[*1]+: yes; -: none. [*2]sEPSC frequency increases produced by eugenol, zingerone, carvacrol and thymol resulted from the activation of TRPA1, but not TRPV1, channels (see [6–8,10]). [*3]Outward currents produced by eugenol, carvacrol and thymol were not mediated by TRPA1 or TRPV1 channels (see [6–8]).

TRPA1 channels are thought to be a target for the treatment of pain [3], the present findings also may serve to reveal chemical structures necessary for activation of TRPA1 channels in the SG.

Many endogenous analgesics produce an outward current at −70 mV and, thus, hyperpolarize membranes of SG neurons, resulting in a decrease in the membrane excitability of these neurons [2]. However, activation of central terminal TRPA1 channels increases spontaneous release of L-glutamate onto SG neurons, leading to increased membrane excitability of these neurons, an effect opposite to that of hyperpolarization. Of the plant-derived compounds tested in the present study, only *p*-cymene produced an outward current in a portion of SG neurons examined without changing sEPSC frequency. This action could contribute, at least in part, to the antinociception produced by intraperitoneal administration of *p*-cymene (which is lipophilic and, thus, permeable to the brain-blood barrier, resulting in activity on CNS neurons) in mice [20].

In conclusion, a chemical moiety (such as $—CH_2CH{=}CH_2$, $—(CH_2)_2COCH_3$ or —OH) bound to the benzene ring of plant-derived compounds is needed to produce an outward current (membrane hyperpolarization) or TRPA1 activation in the SG. This information provides insight for the development of new analgesics based on plant-derived compounds.

Conflicts of interest

The authors have no conflicts in interest to disclose.

Author contributions

Study conception and design: C.W., Y.T., T.F. and E.K.
Acquisition of data: C.W., Y.T. and T.F.
Analysis and interpretation: C.W., Y.T., T.F. and E.K.
Drafting of manuscript: C.W., Y.T., T.F. and E.K.
All authors provided final approval of the final version of the manuscript.

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