Zingerone enhances glutamatergic spontaneous excitatory transmission by activating TRPA1 but not TRPV1 channels in the adult rat substantia gelatinosa

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Yue HY, Jiang CY, Fujita T, Kumamoto E. Zingerone enhances glutamatergic spontaneous excitatory transmission by activating TRPA1 but not TRPV1 channels in the adult rat substantia gelatinosa. J Neurophysiol 110: 658–671, 2013. First published May 8, 2013; doi:10.1152/jn.00754.2012.—Transient receptor potential (TRP) channels such as TRP vanilloid-1 (TRPV1) and TRP ankyrin-1 (TRPA1) channels play an important role in transmitting nociceptive information to the central nervous system through primary afferent neurons from the periphery (Tominaga 2007). TRPV1 channels located in the peripheral terminals of the primary afferent neurons are activated by noxious hot temperature, protons, and vanilloids (Caterina et al. 1997; Caterina and Julius 2001), whereas TRPA1 channels in the peripheral terminals of the neurons are activated by noxious cold temperature, mustard oil, cinnamon oil, and garlic (Jordt et al. 2004; Nilius and Voets 2005; Story et al. 2003). The activation of the peripheral TRP channels depolarizes membrane in the terminals, resulting in the production of action potentials. On the other hand, the activation of the TRP channels in the central terminals results in the enhancement of the spontaneous release of 1-glutamate to superficial medullary dorsal horn (Jennings et al. 2003; Sun et al. 2009) and spinal substantia gelatinosa (SG; lamina II of Rexed) neurons (Baccei et al. 2003; Jiang et al. 2009; Kosugi et al. 2007; Morisset and Urban 2001; Uta et al. 2010; Wrigley et al. 2009; Yang et al. 1998). Taking into consideration that the SG neurons play a pivotal role in regulating nociceptive transmission from the periphery (Willis and Coggeshall 1991), it is possible that the activation of the central TRP channels is involved in modulating the transmission. There is much evidence to support this idea. For instance, peripheral inflammation induced by intraplantar injection of complete Freund’s adjuvant upregulated TRPV1 channels involved in enhancing spontaneous excitatory transmission in rat SG neurons (Lappin et al. 2006) and overexpressed TRPA1 channels in mouse spinal cord and dorsal root ganglia (DRG; da Costa et al. 2010). Intrathecal administration of TRPV1 and TRPA1 antagonists reversed hyperalgesia in rodent models of neuropathic pain (da Costa et al. 2010; Watabiki et al. 2011). Many of the properties of the TRP channels have been examined in the cell body of the primary afferent neuron. We have suggested that a local anesthetic, lidocaine, which acts on TRPV1 channels (Leffler et al. 2008) and to a lesser extent on TRPA1 channels (Leffler et al. 2011) in the cell body of the primary afferent neuron, activates TRPA1 but not TRPV1 channels in the neuron’s central terminal (Piao et al. 2009). The central TRPV1 channel was activated by piperine (a pungent component of black pepper; Szallasi 2005) but not olvanil (the synthetic oleic acid homolog of capsaicin; Yang et al. 2011), both of which compounds activated TRPV1 channels in the cell body of the primary afferent neuron (Liu and Simon 1996; Liu et al. 1997). A vanilloid compound, eugenol, that reportedly activated TRPV1 channels in the cell body (Yang et al. 2003) was shown to activate the central TRPA1 but not TRPV1 channels (Inoue et al. 2007; Morisset and Urban 2001; Uta et al. 2010; Wrigley et al. 2009; Yang et al. 1998). Taking into consideration that the cell body and central terminal of the primary afferent neuron have properties different from each other. To further address this issue, we examined the effect of zingerone [4-(4-hydroxy-3-methoxyphenyl)-2-butanoic acid; a pungent component of ginger, rhizomes of Zingiber officinale Roscoe; Langner et al. 1998], which is a vanilloid compound and

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activates TRPV1 channels in the cell body of the primary afferent neuron (Liu and Simon 1996; Liu et al. 2000), on glutamatergic excitatory and GABAergic inhibitory transmissions in SG neurons.

METHODS

This study was approved by Saga University Animal Care and Use Committee, and was conducted in accordance with the “Guiding Principles for the Care and Use of Animals in the Field of Physiological Science” of the Physiological Society of Japan. All efforts were made to minimize animal suffering and the number of animals used.

Preparation of spinal cord slices. The methods used for obtaining transverse slice preparations of the adult rat spinal cord were similar to those described elsewhere (Fujita et al. 2009; Jiang et al. 2009; Yue et al. 2004, 2005, 2011). Briefly, male Sprague-Dawley rats (6–8 wk old) were anesthetized with urethane (1.2 g/kg ip), and then a lumbarosacral segment (L1–S3) of the spinal cord was removed and placed in cold (2–4°C) Krebs solution preequilibrated with 95% O₂ and 5% CO₂. After all ventral and dorsal roots were cut, the pia-arachnoid membrane was removed. The spinal cord was placed in a shallow groove formed in an agar block, and this was mounted on a stage in a microslicer (DTK-1000; Dousaka, Kyoto, Japan), which was filled with preoxygenated cold Krebs solution; a 600-μm-thick transverse slice was then cut. In some experiments, adult rat spinal cord slice that retained an attached dorsal root was obtained, as reported previously (Fujita et al. 2009; Yue et al. 2011). The slice was placed on a nylon mesh in the recording chamber and was then completely submerged and superfused at a rate of 15–20 ml/min with Krebs solution that was saturated with 95% O₂ and 5% CO₂ and maintained at 36 ± 1°C. The composition of Krebs solution used was (in mM) 117 NaCl, 3.6 KCl, 2.5 CaCl₂, 1.2 MgCl₂, 5 EGTA, 5 HEPES, 5 Mg-ATP, and 5 tetraethyl-ammonium (TEA)-Cl (pH 7.2). The former and latter solutions were used to record excitatory (EPSCs) and inhibitory postsynaptic potentials.

Electrophysiological recordings from SG neurons. SG neurons were identified by their location under a binocular microscope with light transmitted from below; the SG was easily discernible as a relatively translucent band running from the medial site of the dorsal horn across it, around the apex, and down the lateral site. Whole cell voltage-clamp recordings were made from the SG neurons by using a patch pipette fabricated from thin-walled, fiber-filled capillary (1.5-mm outer diameter) with a resistance of 8–15 MΩ (Fujita et al. 2009; Jiang et al. 2009; Yue et al. 2004, 2005, 2011). The patch pipette was inserted at the center of SG under visual control to avoid recordings from lamina I or III neurons. The patch pipette solutions contained (in mM) either 135 K-glucosate, 5 KCl, 0.5 CaCl₂, 2 MgCl₂, 5 EGTA, 5 HEPES, and 5 Mg-ATP or 110 CsSO₄, 0.5 CaCl₂, 2 MgCl₂, 5 EGTA, 5 HEPES, 5 Mg-ATP, and 5 tetraethyl-ammonium (TEA)-Cl (pH = 7.2). The former and latter solutions were used to record excitatory (EPSCs) and inhibitory postsynaptic currents (IPSCs), respectively. EPSCs were recorded at a holding potential (Vₘ) of −70 mV where no IPSCs were observed, since the reversal potential for IPSCs was near −70 mV. On the other hand, IPSCs were observed at a Vₘ of 0 mV where EPSCs were invisible, since the reversal potential for EPSCs was close to 0 mV, Cs⁺ and TEA were added to inhibit K⁺ channels located in the recorded SG neurons and thus to easily shift Vₘ to 0 mV from resting membrane potentials.

Aβ-fiber and C-fiber evoked EPSCs were elicited by stimulating the dorsal root, as mentioned previously (Fujita et al. 2009; Yue et al. 2011). In brief, the stimulation was performed by using a suction electrode with a constant current source of pulse at a frequency of 0.1 Hz unless otherwise mentioned. The strength of stimulus (duration: 0.1 ms) used was 1.2 times a threshold to elicit EPSCs to prevent a conduction block of action potentials in the dorsal root. The fiber-evoked EPSCs were distinguished from each other on the basis of the minimal stimulus strength sufficient to elicit the EPSCs and the latency of the EPSCs. C-fiber EPSCs required much larger stimulus intensity for the activation than Aβ-fiber EPSCs, and Aβ-fiber EPSCs had a shorter latency than C-fiber EPSCs. Aβ-fiber EPSCs were judged to be monosynaptic when the latency remained constant and there was no failure during stimulation at 20 Hz for 1 s, whereas C-fiber EPSCs were judged to be such when failures did not occur during repetitive stimulation at 1 Hz for 20 s, as was done previously (Fujita et al. 2009; Yue et al. 2011). Conduction velocities (CVs) of the afferent fibers were calculated from the latency of monosynaptic EPSC and the length of dorsal root (see Fujita et al. 2009; Yue et al. 2011).

Signals were acquired using an EPC-7 amplifier (HEKA, Lambrecht, Germany) or an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA). Currents obtained in the voltage-clamp mode were low-pass filtered at 3 kHz and digitized at 333 kHz with an analog-to-digital converter (Digidata 1200; Molecular Devices). The data were stored and analyzed with a personal computer using pCLAMP 6.0 software (Molecular Devices). The program (AxoGraph 4.0; Molecular Devices) used for analyzing spontaneous EPSCs (sEPSCs) detected spontaneous events if the difference between the baseline and a following current value exceeds a given threshold of 5 pA and the peak is separated from an adjacent peak by an intervening valley that is deeper than 50% of the adjacent peak. The validity of whether sEPSCs were accurately detected by the program was confirmed by measuring visually individual sEPSCs in a fast time scale on a computer screen in several cases. Spontaneous IPSCs (sIPSCs) were detected and analyzed using Mini Analysis Program; the results so obtained were not so different from those obtained using AxoGraph. Numerical data are presented as means ± SE. Statistical significance was determined as P < 0.05 using either (paired or unpaired) Student’s t-test (unless otherwise noted) or the Kolmogorov-Smirnov test. In all cases, n refers to the number of neurons studied.

Application of drugs. Drugs were applied by perfusing a solution containing drugs of a known concentration without an alteration in the perfusion rate and temperature. The solution in the recording chamber having a volume of 0.5 ml was completely replaced within 15 s. The drugs used were capsaicin, allyl isothiocyanate (AITC), tetrodotoxin (TTX; Wako, Osaka, Japan), zingerone, ruthenium red, capsazepine, HC-030031, 2-aminoethoxydiphenyl borate (2-APB), dantradole sodium salt, strychnine nitrate (Sigma-Aldrich, St. Louis, MO), 6-cyano-7-nitroquininaline-2,3-dione (CNQX), and α-2-amino-5-phosphonovaleric acid (APV; Tocris Cookson, Bristol, UK). These drugs (except for AITC, capsazepine, HC-030031, CNQX, 2-APB, and dantradole, for which dimethyl sulfoxide was used as solvent) were first dissolved in distilled water at 1,000 times the concentration to be used and then stored at −25°C. The stock solution was diluted to the desired concentration in Krebs solution immediately before use. The osmotic pressure of nominally Ca²⁺-, Mg²⁺-, and Na⁺-free, high-Mg²⁺ (5 mM) Krebs solution was adjusted by lowering the Na⁺ concentration.

RESULTS

Whole cell patch-clamp recordings were made from a total of 153 SG neurons. Stable recordings could be obtained from slices maintained in vitro for more than 10 h, and recordings were made from single SG neurons for up to 3 h. All SG neurons tested had resting membrane potentials lower than −60 mV (when measured in the current-clamp mode) and exhibited sEPSCs at the Vₘ of −70 mV (Fujita et al. 2009; Liu et al. 2008; Yue et al. 2005, 2011). The sEPSCs were completely blocked by the non-NMDA receptor antagonist CNQX.
(10 μM) and were not affected by the voltage-gated Na\(^+\)-channel blocker TTX (0.5 μM) (Fujita and Kumamoto 2006; Fujita et al. 2009; Inoue et al. 2012; Jiang et al. 2009; Kosugi et al. 2007; Yang et al. 1998; Yue et al. 2005, 2011). Thus sEPSCs were not contaminated by sIPSCs and occurred without spike propagation from the soma of presynaptic TTX-sensitive neurons. Different from the latter result, sEPSCs in SG neurons of young (12–29 days old) rats were smaller in frequency and amplitude than those in the presence of TTX (Haseneder et al. 2004; Lappin et al. 2006). This difference in TTX sensitivity will be due to a distinction in age between the rats used.

Effects of zingerone on glutamatergic spontaneous excitatory transmission in SG neurons. Zingerone (2 mM) superfused for 2 min enhanced spontaneous excitatory transmission in an SG neuron (Fig. 1A). The sEPSC frequency increased gradually over time, peaking ~2.5 min after the onset of zingerone addition; this facilitation was accompanied by a small increase in sEPSC amplitude (Fig. 1B). Such an increase in sEPSC frequency and amplitude was seen in all neurons examined (n = 63). This increase in sEPSC frequency subsided within 10 min after zingerone washout. The sEPSC frequency and amplitude ~2.5 min after zingerone addition were, respectively, 360 ± 34% (n = 63; P < 0.05) and 126 ± 3% (n = 63; P < 0.05) of those before its addition (control; 8.9 ± 0.9 Hz and 9.4 ± 0.3 pA). Zingerone significantly increased the proportion of sEPSCs with a shorter interevent interval and a larger amplitude (Fig. 1C); this effect was confirmed in three other neurons. Such a change in sEPSC frequency and amplitude was accompanied by an inward current with the peak amplitude of 14.8 ± 2.0 pA (n = 28) in 44% of the neurons examined (n = 63; for example see Fig. 1A).

Spontaneous excitatory transmission enhancement produced by the TRPV1 agonists capsaicin or resiniferatoxin in SG neurons shows slow recovery from desensitization (Jiang et al. 2009; Yang et al. 1998). We therefore examined how repeated application of zingerone (2 mM) affects spontaneous excitatory transmission. As shown in Fig. 2, Aa and Ab, the zingerone action was repeated at an interval of 20 min. The initial and second zingerone treatments produced similar sEPSC frequency and amplitude increases (n = 6; Fig. 2Ac), indicating that sEPSC frequency changes were not consistent with desensitization from TRPV1 activation. As noted from Fig. 2, Aa and Ab, the zingerone-induced inward current was also repeated at an interval of 20 min. In five of the six neurons, the current amplitudes were not different from each other (P > 0.05; Fig. 2Ad). The remaining neuron did not exhibit any inward current.

Figure 2Bd demonstrates the chart recordings of the action of zingerone at the concentrations of 0.5, 1 and 5 mM on excitatory transmission, as examined in the same neuron. The sEPSC frequency increase produced by zingerone became to be large in extent with an increase in concentration. Figure 2Bb demonstrates the concentration-response relationship for changes in sEPSC frequency and amplitude produced by zingerone in a range of 0.1–5 mM. Analysis based on the Hill equation showed that the effective concentration of zingerone for half-maximal effect (EC\(_{50}\)) in increasing sEPSC frequency is 1.3 mM. Zingerone at 0.1 mM did not change holding currents in all neurons examined (n = 6), but this drug at concentrations higher than 0.2 mM produced an inward current in ~40% of the neurons tested. Neurons exhibiting the inward current at 0.2, 0.5, 1 and 5 mM were, respectively, 43, 33, 36, and 43% of those examined (n = 7, 9, 11, and 14, respectively). Their peak amplitudes were 11.0 ± 1.8 pA (n = 3),
The zingerone action was not affected by TTX (0.5 μM), as shown in Fig. 3, Aa and Ab. sEPSC frequency and amplitude increases produced by zingerone in the presence of TTX were not different from those in the absence of TTX (Fig. 3Ac), indicating that zingerone action is not due to an increase in neuronal activity. TTX also did not affect the inward current produced by zingerone (Fig. 3, Aa and Ab). In seven of the eight neurons examined, zingerone produced an inward current; in the absence and presence of TTX, the currents were not significantly different from each other (P > 0.05; Fig. 3C). The remaining neuron did not respond to zingerone. Figure 3B demonstrates the effect of zingerone (2 mM) on spontaneous excitatory transmission in the absence and presence of a mixture of CNQX (10 μM) and the NMDA receptor antagonist APV (50 μM). The antagonists blocked all sEPSCs before and after zingerone addition while not affecting the inward current produced by zingerone. The peak amplitudes of the inward currents in the absence and presence of both CNQX and APV were not significantly different from each other (Fig. 3C).

Moreover, the zingerone action persisted in a voltage-gated Ca2+-channel blocker La3+ (30 μM; Gu and MacDermott 1997)-containing or nominally Ca2+-free, high-Mg2+- (5 mM) Krebs solution (Fig. 4, Aa and Ab). sEPSC frequency and amplitude ~2.5 min after zingerone (2 mM) addition in La3+-containing or Ca2+-free solutions, relative to those just before zingerone application, were not significantly different from those in normal Krebs solution (Fig. 4Ac). Zingerone-induced inward currents also persisted in La3+-containing and Ca2+-free solutions where Ca2+ influx was inhibited; neurons exhibiting the inward current in these solutions were, respectively, 60% and 60% of those examined (n = 5 and 5, respectively). Their peak amplitudes were not so different from those in normal Krebs solution (Fig. 4Ad). Altogether, it is possible that zingerone activity is not mediated by neurotransmitters/neuromodulators released as a result of zingerone action and thus a direct action of this drug. As noted from Fig. 4, Aa and Ab, superfusing La3+-containing or Ca2+-free solution itself reduced sEPSC frequency [76 ± 5% (n = 5; P < 0.05) and 84 ± 3% (n = 5; P < 0.05) of control, respectively], as

Fig. 2. The excitatory transmission enhancement produced by zingerone is repeated and concentration dependent. A, a and b: chart recordings of sEPSCs under the 1st application of zingerone (2 mM; a) and 20 min after its washout under the 2nd application of zingerone (2 mM; b), obtained from the same neuron. Note that the inward current produced by zingerone is also repeated. A, c and d: sEPSC frequency and amplitude ~2.5 min after zingerone (2 mM) addition, relative to those just before its addition [1st: 6.4 ± 1.1 Hz, 10.3 ± 0.8 pA (n = 6); 2nd: 6.2 ± 0.8 Hz, 10.2 ± 0.6 pA (n = 6); c], and peak inward current amplitudes (n = 5; d) under the action of the 1st and 2nd applications. *P < 0.05; n.s., not significant. Bc: chart recordings of sEPSCs in the absence and presence of zingerone at concentrations of 0.5, 1, and 5 mM, obtained from the same neuron. Bb: the frequency and amplitude of sEPSC under the action of zingerone, relative to those before its superfusion, plotted against the logarithm of zingerone concentration. This zingerone effect was measured for 0.5 min at ~2.5 min after the beginning of its superfusion. Values in parentheses denote the numbers of neurons examined. Each point with vertical bars represents the mean and SE. If the SE of the values is smaller than the size of the symbol, the vertical bar is not shown. The concentration-response curve for sEPSC frequency was drawn according to the Hill equation (EC50 = 1.3 mM; Hill coefficient = 2.8). Vm = −70 mV.
B, the ability of capsazepine (10 μM) to inhibit TRPV1 action. The TRPV1 antagonist capsazepine [10 μM, a concentration sufficient to inhibit a 2 μM capsaicin response in adult rat SG neurons (see below and Yang et al. 1998; for a similar result in rat spinal deep dorsal horn neurons, see Kim et al. 2009)] did not block the ability of zingerone to increase sEPSC frequency and amplitude in all neurons examined (Fig. 5, Aa, Ab, and Ad), indicating no involvement of TRPV1 channels. Capsazepine itself did not affect sEPSC frequency and amplitude (99 ± 3% and 102 ± 5%, respectively, of control; n = 6; each P > 0.05). Capsazepine also did not affect the inward current produced by zingerone (Fig. 5, Aa and Ab). Zingerone-induced inward current in the presence of capsazepine was seen in five of the six neurons tested. The peak amplitudes of the inward currents in the absence and presence of capsazepine were not significantly different from each other (Fig. 5Ae). As shown in Fig. 5B, the ability of capsazepine (10 μM) to inhibit TRPV1 activation was confirmed in this study. In the presence of this antagonist, sEPSC frequency and amplitude were, respectively,
157 ± 43% and 108 ± 7% (n = 3; each P > 0.05) of control ~2 min after addition of the TRPV1 agonist capsaicin (2 μM), and there was an inward current with a peak amplitude of 6.5 ± 1.6 pA (n = 3); these values were smaller than those in the absence of capsazepine (see below; Jiang et al. 2009; Yang et al. 1998, 2000).

In contrast, the nonspecific TRP antagonist ruthenium red (300 μM) blocked the facilitatory effect of zingerone (Fig. 5, Ac and Ad), where ruthenium red itself did not affect spontaneous excitatory transmission (also see Inoue et al. 2012; Piao et al. 2009). Moreover, zingerone-induced inward currents in the presence of ruthenium red were significantly smaller in
amplitude than those in the absence of this antagonist (Fig. 5Ae). These results indicate that zingerone activity is due to the activation of TRP channels other than TRPV1 channels.

Since TRPA1 activation enhanced spontaneous excitatory transmission in SG neurons (Inoue et al. 2012; Kosugi et al. 2007; Piao et al. 2009; Uta et al. 2010; Wrigley et al. 2009), we examined how the TRPA1 antagonist HC-030031 (50 μM; McNamara et al. 2007) affects excitatory transmission enhancement produced by zingerone. HC-030031 inhibited the zingerone effect (Fig. 5C). The sEPSC frequency and ampli-
tude percentage values under the action of zingerone were significantly smaller than those in the absence of HC-030031 in the same neuron (Fig. 5, Ca and Cb). Moreover, zingerone-induced inward currents in the presence of HC-030031 were significantly smaller in amplitude than those in the absence of this antagonist (Fig. 5, Ca and Cc). These results indicate an involvement of TRPA1 channels.

To confirm the involvement of TRPA1 but not TRPV1 channels in the zingerone-induced excitatory transmission enhancement, we further investigated whether zingerone activity is affected by the TRPA1 agonist AITC and also by capsaicin. In a neuron sensitive to AITC (100 μM), zingerone (2 mM) increased sEPSC frequency and amplitude ~25 min after AITC washout (Fig. 6A); similar results were obtained from three other neurons (Fig. 6E). This zingerone activity was not significantly different from that without the pretreatment with AITC (see Figs. 2Bb and 6E). Next, we examined the effects of the coapplication of AITC (100 μM) and zingerone (2 mM) on spontaneous excitatory transmission. Although this coapplication ~25 min after AITC (100 μM) superfusion also increased sEPSC frequency and amplitude, these increases were not different in extent from those produced by AITC only (see Fig. 6B); similar results were obtained from four other neurons (Fig. 6E). This result suggests that zingerone and AITC may activate the same type of TRP channel.

As shown in Fig. 6C, the facilitatory action of capsaicin exhibits a slow recovery from desensitization in SG neurons (Jiang et al. 2009; Yang et al. 1998). sEPSC frequency and amplitude 3 min after the first addition of capsaicin, relative to those just before its application, were larger than those obtained ~3 min after its second addition (Fig. 6E). In a neuron where capsaicin (2 μM) enhanced excitatory transmission, the transmission was facilitated by zingerone (2 mM) ~15 min after capsaicin washout (Fig. 6Da). Figure 6E demonstrates sEPSC frequency and amplitude ~2–2.5 min after zingerone or capsaicin addition, relative to those just before its application. Zingerone (2nd application) produced inward currents with an amplitude of 7.8 ± 0.4 pA (n = 3), whereas capsaicin (1st application) had no response (n = 1) or induced inward currents (amplitude: 6.2 and 22.5 pA). On the other hand, capsaicin (2 μM) was effective in enhancing excitatory transmission (sEPSC frequency: 529% and 270% of control 2 min after capsaicin application) some 15 min after zingerone (2 mM) facilitated the transmission (sEPSC frequency: 448% and 123% of control 2.5 min after zingerone application; n = 2; for example see Fig. 6Db). In a neuron where capsaicin (2nd application) did not change holding current, zingerone (1st application) produced an inward current with an amplitude of 13.5 pA; in another neuron, they induced inward currents with amplitudes of 22.5 and 17.5 pA, respectively. Thus there was no interaction between the effects of zingerone and capsaicin on excitatory transmission. Altogether, these results indicate an involvement of TRPA1 but not TRPV1 channels.

**Effect of zingerone on dorsal root-evoked monosynaptic excitatory transmission in SG neurons.** Since TRPV1 and TRPA1 activations inhibited monosynthetically evoked excitatory transmission in adult rat SG neurons (Uta et al. 2009; Yang et al. 1999), we examined whether zingerone (2 mM) affects monosynaptic Aδ-fiber and C-fiber EPSCs in the SG neurons. Stimulating the dorsal root with a strength of more than 36 μA (sufficient to recruit Aδ-fibers) elicited in some neurons monosynaptic EPSCs that displayed no failure and no change in latency when examined at 20 Hz (see Fig. 7A, left). CV values estimated from the latency of EPSC averaged 7.9 ± 0.5 m/s (range: 7.3–8.9 m/s; n = 3). Monosynaptic Aδ-fiber EPSCs evoked at 0.1 Hz had a mean amplitude of 134 ± 28 pA (range: 80–175 pA). On the other hand, stimuli with a strength larger than 590 μA (enough to activate C-fibers) evoked in some neurons monosynaptic EPSCs that had no failures albeit a variety in the latency when tested at 1 Hz (see Fig. 7A, right). The monosynaptic EPSCs had an average CV of 0.49 ± 0.03 m/s (range: 0.43–0.56 m/s; n = 3). Monosynaptic C-fiber EPSCs evoked at 0.1 Hz had a mean amplitude of 51 ± 5 pA (range: 45–66 pA; n = 4). As shown in Fig. 7B and C, monosynaptic Aδ-fiber and C-fiber EPSC amplitudes were reduced by zingerone in a reversible manner. These results were confirmed in two and three other neurons, respectively; the monosynaptic Aδ-fiber and C-fiber EPSC amplitudes were reduced from 134 ± 28 to 100 ± 26 pA (25 ± 9%; n = 3) and from 51 ± 5 to 31 ± 3 pA (37 ± 10%; n = 4), respectively, and 6 min after washout of zingerone, Aδ-fiber and C-fiber EPSC amplitudes recovered to 120 ± 26 pA (n = 3) and 44 ± 5 pA (n = 4), respectively. The Aδ-fiber and C-fiber EPSC amplitude reductions were not significantly different in extent from each other (P > 0.05).

**Effect of zingerone on GABAergic spontaneous inhibitory transmission in SG neurons.** Because AITC (100 μM) enhances spontaneous inhibitory transmission in adult rat SG neurons (Kosugi et al. 2007), we next examined how zingerone (2 mM) affects spontaneous GABAergic transmission in the SG neurons. As shown in Fig. 8A, zingerone superfused for 2 min enhanced spontaneous GABAergic transmission recorded in the presence of the glycine receptor antagonist strychnine (1 μM). Such an enhancement was observed in 4 of the 5 neurons tested; sIPSC frequency and amplitude were 253 ± 46% (P < 0.05) and 175 ± 15% (P < 0.05) of control (7.4 ± 1.7 Hz and 10.2 ± 0.9 pA; n = 4), respectively, ~2.5 min after zingerone addition. As shown for AITC actions (Kosugi et al. 2007), such a facilitatory action was not seen in the presence of TTX (0.5 μM; see Fig. 8B), where GABAergic sIPSC frequency and amplitude were 108 ± 21% (P > 0.05) and 96 ± 5% (P > 0.05) of control (2.2 ± 0.3 Hz and 8.5 ± 0.2 pA; n = 3), respectively, ~2.5 min after zingerone addition.

Fig. 5. The excitatory transmission enhancement produced by zingerone (2 mM) is inhibited by nonselective transient receptor potential (TRP) antagonist ruthenium red (RR) and TRPA1 antagonist HC-030031 but not by TRPV1 antagonist capsazepine (Capz). A, a–c: recordings of sEPSCs under the action of zingerone in the absence (a) and presence of Capz (10 μM; b) or RR (300 μM; c), obtained from the same neuron at an interval of 20 min. Note that inward current produced by zingerone is resistant to Capz while inhibited by RR. A, a and d: e: sEPSC frequency and amplitude ~2.5 min after zingerone addition, relative to those just before its addition [Capz: 7.1 ± 4.0 Hz and 9.4 ± 1.3 pA (n = 6); RR: 7.2 ± 2.6 Hz and 8.9 ± 1.0 pA (n = 6); d]: and peak inward current amplitudes in the absence and presence of Capz (n = 6) or RR (n = 6; e). *P < 0.05. B: recording of sEPSCs and inward current under the action of capsaicin (2 μM) in the presence of Capz (10 μM). Ca: recordings of sEPSCs under the action of zingerone (left) and presence of HC-030031 (50 μM; right), obtained from the same neuron at an interval of 20 min. Note that inward current produced by zingerone is inhibited in the presence of HC-030031. C, b and c: sEPSC frequency and amplitude ~2.5 min after zingerone addition, relative to those just before its addition [8.4 ± 1.6 Hz and 9.2 ± 0.9 pA (n = 4); b], and peak inward current amplitudes in the absence and presence of HC-030031 (n = 4; c). *P < 0.05. V_H = −70 mV.

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We found that zingerone increases sEPSC frequency with a small increase in its amplitude in all SG neurons examined, albeit this extent is variable among the neurons. On the other hand, in about half of the neurons tested, this increase was accompanied by an inward current at $-70 \text{ mV}$. This variability may be due to the fact that the SG comprises a heterogeneous cell group (Grudt and Perl 2002). Consistent with the sEPSC frequency increase, zingerone shifted the distribution of the interevent interval of sEPSC to a short one, indicating that this...

Fig. 6. The excitatory transmission enhancement produced by zingerone (2 mM) is occluded by TRPA1 agonist allyl isothiocyanate (AITC; 100 μM) but not capsaicin (2 μM). A: recordings of sEPSCs in the absence and presence of AITC (left) or zingerone (right). B: recordings of sEPSCs in the absence and presence of AITC without (left) or with zingerone (right). C: recordings of sEPSCs under the 1st (left) and 2nd applications of capsicain (right). Note that the 1st but not 2nd application of capsicain produces an inward current. In each of A, B and C, the right recording was obtained 25 min after the left recording, obtained from the same neuron. D: recordings of sEPSCs in the absence and presence of zingerone or capsicain. D, a and b: zingerone was applied 15 min after capsicain washout (a); capsicain was applied 15 min after zingerone washout (b). E: sEPSC frequency and amplitude under the action of AITC ($n = 4$) vs. zingerone ($n = 4$); AITC ($n = 5$) vs. both AITC and zingerone ($n = 5$); the 1st ($n = 3$) vs. 2nd ($n = 3$) application of capsicain; and capsicain ($n = 3$) vs. zingerone ($n = 3$), all relative to those just before each superfusion. The effect of AITC only and the coapplication effect of AITC and zingerone were measured 4.5 min after each addition. *P < 0.05. $V_{\text{H}} = -70 \text{ mV}$. Caps, capsaicin; Zing, zingerone.

DISCUSSION

We found that zingerone increases sEPSC frequency with a small increase in its amplitude in all SG neurons examined, albeit this extent is variable among the neurons. On the other hand, in about half of the neurons tested, this increase was accompanied by an inward current at $-70 \text{ mV}$. This variability may be due to the fact that the SG comprises a heterogeneous cell group (Grudt and Perl 2002). Consistent with the sEPSC frequency increase, zingerone shifted the distribution of the interevent interval of sEPSC to a short one, indicating that this...
The sEPSC frequency increase and inward current were resistant to voltage-gated Na\(^+\) and Ca\(^{2+}\)-channel blockers (TTX and La\(^{3+}\), respectively) and persisted in Ca\(^{2+}\)-free solution, and the inward current was further unaffected by glutamate receptor antagonists (CNQX and APV), suggesting a direct action of zingerone. The frequency increase was enhanced with an increase in zingerone concentration with an EC\(_{50}\) value of 1.3 mM, whereas the inward current did not exhibit a clear concentration dependency. Liu and Simon (1996) have reported that zingerone produces an inward current sensitive to the TRPV1 antagonist capsazepine in cultured rat trigeminal ganglion (TG) neurons. The zingerone-induced sEPSC frequency increase and inward current in the present study were, however, unaffected by capsazepine, which blocked the facilitatory action of spontaneous excitatory transmission produced by the TRPV1 agonists capsaicin and resiniferatoxin in rat SG neurons (Jiang et al. 2009; Yang et al. 1998). No involvement of TRPV1 channels in the zingerone actions in SG neurons is supported by the observations that there was not an interaction between the facilitatory actions of zingerone and capsaicin on excitatory transmission and that unlike capsaicin, zingerone enhanced spontaneous GABAergic transmission (see Yang et al. 1998). Alternatively, the zingerone actions were repeated at an interval of 20 min, an observation different from capsaicin-induced TRPV1 activation, which did not recover 20 min after its superfusion in the SG (Jiang et al. 2009; Yang et al. 1998). These observations were different from those in rat TG neurons in that there was a cross-desensitization between zingerone and capsaicin responses and that a repeated application of zingerone exhibited a desensitization (Liu et al. 2000; Liu and Simon 1996). On the other hand, the zingerone actions in rat SG neurons were blocked by the nonselective TRP antagonist ruthenium red, indicating an involvement of TRP channels other than TRPV1 channels.

A repeated action, similar to that of zingerone, has been observed in the presynaptic actions of AITC, lidocaine, and eugenol, all of which are thought to activate TRPA1 channels in the SG (Inoue et al. 2012; Kosugi et al. 2007; Piao et al. 2009). The zingerone action in the present study was inhibited by the TRPA1 antagonist HC-030031, indicating an involvement of TRPA1 channels. This idea is supported by the observation that zingerone as well as AITC enhances spontaneous inhibitory transmission. AITC inhibits the facilitatory action of zingerone on excitatory transmission, indicating an occlusion at the level of TRPA1 channel, although the possibility cannot be ruled out that this inhibition occurs at the level of downstream after channel activation such as release machinery. Considering that IC\(_{50}\) concentration for HC-030031 in inhibiting human TRPA1 activation by AITC is 0.7 \(\mu\)M (McNamara et al. 2007), the concentration of HC-030031 (50 \(\mu\)M) used in the present study may have been quite high.

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μM, appeared to be necessary to inhibit TRPA1 activation in the rat spinal or medullary dorsal horn (Cho et al. 2012; Uta et al. 2010).

The rat DRG neurons express TRPA1 mRNAs and proteins (Kim et al. 2010; Kobayashi et al. 2005; Story et al. 2003). Although rat TG neurons responded to AITC (Akopian et al. 2007), zingerone at a high concentration, such as 30 mM, produced only a response sensitive to capsaicine (Liu and Simon 1996). Zingerone may activate different types of TRP channel between the cell body and central terminal of the primary afferent neuron. There may be a TRPV1 splice variant (for example, see Tian et al. 2006) or a TRPA1 channel modulated by second messengers (Dai et al. 2007; Schmidt et al. 2009; Wang et al. 2008), which is activated by zingerone in a manner sensitive to the TRPA1 but not TRPV1 antagonist, in the central terminal of primary afferent neurons. More experiments such as recordings from the cell body of DRG neurons having monosynaptic inputs to SG neurons will be required to confirm our idea that TRP channels differ in property between the central terminal and cell body of DRG neurons.

Although HC-030031 has been reported to be not only a TRPA1 but also a TRPV1 antagonist (Iwasaki et al. 2009), it is unlikely that the HC-030031 action in the present study is attributed to TRPV1 inhibition, because the actions of zingerone as well as AITC, lidocaine, and eugenol in SG neurons are unaffected by capsaicine (see above; Inoue et al. 2012; Kosugi et al. 2007; Piao et al. 2009). The EC$_{50}$ value (1.3 mM) for zingerone in increasing sEPSC frequency in SG neurons was smaller by about 10-fold than that (15 mM) for TRPV1 activation by zingerone in rat TG neurons (Liu and Simon 1996). The present study demonstrated for the first time that zingerone increases sEPSC frequency in SG neurons by TRPA1 activation. The possibility cannot be ruled out that a part of this zingerone activity is mediated by an impurity contained in the zingerone purchased (purity: ≥96%). The TRPA1 activation requires zingerone at concentrations >1 mM, which contain an impurity that may activate other types of TRP channel, such as TRP melastatin-8 (TRPM8) and TRPV2 channels. TRPM8 activation also increases sEPSC frequency in SG neurons was smaller by about 10-fold than that (15 mM) for TRPV1 activation by zingerone in rat TG neurons (Liu and Simon 1996). The present study demonstrated for the first time that zingerone increases sEPSC frequency in SG neurons by TRPA1 activation. The possibility cannot be ruled out that a part of this zingerone activity is mediated by an impurity contained in the zingerone purchased (purity: ≥96%).

The peak amplitude of zingerone-induced inward current in the present study was unchanged by glutamate receptor antagonists, an observation different from AITC-induced current, which disappeared in the presence of glutamate receptor antagonists. The AITC current was mediated by an excess of Aδ-fiber and C-fiber EPSC amplitude reductions produced by zingerone, an observation different from the actions of capsaicin and cinnamaldehyde, which reduced primary afferent C-fiber but not Aδ-fiber EPSC amplitude (Uta et al. 2010; Yang et al. 1999). This issue remains to be further addressed.

A question remains why the central TRPV1 channel is not activated by zingerone, which activates TRPV1 channels in the cell body of primary afferent neurons. The central TRPV1 activation may need zingerone at concentrations higher than those used in the present study, because zingerone (1 mM) produces only a small response (~10% of a maximal response induced by capsaicin) in rat DRG neurons (Dedov et al. 2002). Alternatively, the presynaptic TRPA1 activation may affect the ability of zingerone to activate presynaptic TRP channels. Many of DRG neurons coexpress TRPA1 and TRPV1 channels (Kobayashi et al. 2005), and a TRPA1-TRPV1 channel complex may be formed on the plasma membrane (Staruschenko et al. 2010). This issue remains to be further examined.
release of l-glutamate, as shown in P2X activation at glutamatergic terminals in the brain stem (Shigetomi and Kato, 2004).

The zingerone-induced sEPSC frequency increase in the present study would be due to an increase in intraterminal Ca\(^{2+}\) concentration. It is unlikely that this Ca\(^{2+}\) increase is due to voltage-gated Ca\(^{2+}\)-channel activation following a membrane depolarization produced by zingerone, because the zingerone activity is resistant to the voltage-gated Ca\(^{2+}\)-channel blocker La\(^{3+}\). TRPA1 channel is permeable to Ca\(^{2+}\) (Jordt et al. 2004; Nilius et al. 2011), and thus this channel activation would result in Ca\(^{2+}\) entry from external solution, followed by an increase in intraterminal Ca\(^{2+}\) concentration. TRPA1 activity in Ca\(^{2+}\)-free solution, therefore, would be expected to decrease in extent, but this was not the case in the present study. On the other hand, AITC-induced sEPSC frequency increase was suppressed in Ca\(^{2+}\)-free solution (Kosugi et al. 2007).

TRPA1 activations by zingerone and AITC may be distinct from each other in mode such as desensitization and intracellular Ca\(^{2+}\) mobility. As shown in rat primary afferent neurons, TRPA1 desensitization in nerve terminals may be decreased in extent in Ca\(^{2+}\)-free solution (Akopian et al. 2007; Raisinghani et al. 2011). The decrease in the desensitization of zingerone activity may result in TRPA1 activity increase that overrides the decrease in Ca\(^{2+}\) entry through TRPA1 channels in Ca\(^{2+}\)-free solution. It is possible that desensitization is distinct in extent between zingerone- and AITC-activated TRPA1 responses, because agonist-dependent desensitization has been demonstrated for many types of ligand-gated channel, including TRPA1 and TRPV1 channels (Liu and Simon 1996; Raisinghani et al. 2011). Alternatively, it is likely that intraterminal Ca\(^{2+}\) level increases produced by zingerone and AITC are distinct from each other, because a Ca\(^{2+}\) permeability of TRPA1 channel alters in extent depending on agonists involved in its activation (Karashima et al. 2010; Nilius et al. 2011). Intraterminal Ca\(^{2+}\) level increase by TRPA1 activation may be due to not only Ca\(^{2+}\) entry from external solution but also Ca\(^{2+}\) release from intracellular stores. In support of this idea, TRPV1 activation mobilized Ca\(^{2+}\) from intracellular stores in TRPV1-transfected HEK-293 cells (Marshall et al. 2003). Moreover, TRPV1 activation by capsaicin at synapses in DRG/spinal cord co-cultures prolonged the elevation of intraterminal Ca\(^{2+}\) levels and increased l-glutamate release (Medvedeva et al. 2008). Although intracellular Ca\(^{2+}\) level rise is known to activate phospholipase C, resulting in the production of IP\(_3\) (Ryan et al. 2000), IP\(_3\)-sensitive Ca\(^{2+}\) stores do not appear to be involved in the zingerone activity, because this activity is not inhibited but rather facilitated by the IP\(_3\)-induced Ca\(^{2+}\)-release inhibitor 2-APB. Since 2-APB itself does not almost affect excitatory transmission, this facilitation may be due to a sensitization of TRPA1 channels, as seen for TRPV3 channels (Chung et al. 2004). On the other hand, Ca\(^{2+}\)-induced Ca\(^{2+}\)-release mechanisms appeared to be involved in the presynaptic action of zingerone, because the Ca\(^{2+}\)-induced Ca\(^{2+}\)-release inhibitor dantrolene reduced the extent of sEPSC frequency increase produced by zingerone. Taken together, these findings indicate that a small Ca\(^{2+}\) influx through TRPA1 channels activated by zingerone in nominally Ca\(^{2+}\)-free solution may mobilize Ca\(^{2+}\) from intracellular stores, probably through Ca\(^{2+}\)-induced Ca\(^{2+}\)-release mechanisms, or produce “spotty” Ca\(^{2+}\) microdomains that are highly localized near membrane (Shigetomi et al. 2011), leading to sEPSC frequency increase. The difference in Ca\(^{2+}\) sensitivity between TRPA1 activations by zingerone and AITC remains to be further examined.

In conclusion, zingerone enhances glutamatergic spontaneous excitatory transmission in rat SG neurons; this action is due to the activation of TRPA1 but not TRPV1 channels expressed in the central terminals of primary afferent neurons.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


REFERENCES


GLUTAMATERGIC TRANSMISSION ENHANCEMENT BY ZINGERONE


