Effects of Eugenol on T-type Ca$^{2+}$ Channel Isoforms

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Abbreviations: DMEM, Dulbecco's Modified Eagle Medium; EGTA, ethylene glycol tetraacetic acid; HEK, human embryonic kidney; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IC\textsubscript{50}, the half-maximal inhibitory concentration; TEACl, tetraethylammonium chloride; TG, trigeminal ganglion
ABSTRACT

Eugenol has been used as an analgesic in dentistry. Previous studies have demonstrated that voltage-gated Na⁺ channels and high-voltage-activated Ca²⁺ channels expressed in trigeminal ganglion (TG) neurons sensing dental pain are molecular targets of eugenol for its analgesic effects. However, it has not been investigated whether eugenol can affect T-type Ca²⁺ channels, which are known to be detected in the afferent neurons. In this report, we investigate how eugenol can influence cloned T-type channel isoforms expressed in HEK293 cells, using whole-cell patch clamp. Application of eugenol inhibited Cav3.1, Cav3.2, and Cav3.3 currents in a concentration-dependent manner with IC₅₀ values of 463, 486, and 708 μM, respectively. Eugenol was found to negatively shift the steady-state inactivation curves of the T-type channel isoforms, but it did not shift their activation curves. In addition, eugenol had little effect on the current kinetics of Ca₃.1 and Ca₃.2, but it accelerated the inactivation kinetics of Ca₃.3 currents. Reduction of channel availability enhanced eugenol inhibition sensitivity for Cav3.1 and Cav3.2, but not for Cav3.3. Moreover, eugenol inhibition of T-type channel isoforms was found to be use-dependent. Finally, we show that the T-type currents recorded from rat TG neurons were inhibited by eugenol with a similar potency to Cav3.1 and Cav3.2 isoforms. Taken together, our findings suggest that T-type Ca²⁺ channels are additional...
molecular targets for the pain-relieving effects of eugenol.
INTRODUCTION

Eugenol, the main component in the essential oils extracted from cloves, has been used in dental clinics as an analgesic (Markowitz et al., 1992). Extensive studies to unveil the pain-relieving mechanism of eugenol have found that the chemical potently inhibits diverse ion channels expressed in trigeminal ganglion (TG) neurons that sense dental pain (Lee et al., 2005; Park et al., 2006; Kim et al., 2011). In particular, voltage-activated Na\(^{+}\) channels and high-voltage-activated (HVA) Ca\(^{2+}\) channels have been implicated as eugenol’s molecular targets for lessening pain (Lee et al., 2005; Park et al., 2006). In addition to those ion channels, low-voltage-activated (LVA) T-type channel currents in TG neurons have been detected by electrophysiological recordings (Borgland et al., 2001, 2002; Ikeda and Matsumoto, 2003; Ross et al., 2009). Expression of T-type Ca\(^{2+}\) channels in TG neurons suggests that they might be involved in pain-sensing processes, a prominent role they play in dorsal root ganglion neurons (Bourinet et al., 2005; Nelson et al., 2007; Jagodic et al., 2007, 2008). The activation threshold of T-type channels is generally known to be lower than those of HVA Ca\(^{2+}\) channels as well as Na\(^{+}\) channels (McCormick and Huguenard, 1992; Kim et al., 2001; Llinás and Steriade, 2006). Thus, they could play a pivotal role in initiating depolarizing signals around resting membrane potentials, contributing to the generation of action potential in
the coding of pain signals.

Molecular studies have uncovered that T-type Ca\(^{2+}\) channels are encoded by three different genes (Cav3.1, Cav3.2, and Cav3.3). Electrophysiological characterization has shown that the three isoforms begin to be activated around resting membrane potential. Cav3.1 and Cav3.2 channel currents have been characterized to have fast activating and inactivating kinetics in response to step depolarizing potentials, whereas Cav3.3 has shown considerably slower activating and inactivating kinetics (Perez-Reyes, 2003). Electrophysiological recordings and in situ hybridization detected distinctive physiological roles and expression patterns of the three T-type channel isoforms. Recent experiments on Cav3.1- or Cav3.2-deficient mice further revealed that Cav3.1 is involved in visceral pain and rebound bursting in thalamic neurons (Kim et al., 2001, 2003), while Cav3.2 is involved in peripheral pain processing via dorsal root ganglion neurons (Bourinet et al., 2005) and in maintenance of blood vessel tone (Chen et al., 2003).

Although recordings of T-type channel currents have been reported in TG neurons (Borgland et al., 2001, 2002; Ikeda and Matsumoto, 2003; Ross et al., 2009), no previous studies have determined whether eugenol can inhibit T-type channel currents. Therefore, we examine how eugenol affects three T-type Ca\(^{2+}\) channel isoforms individually expressed in HEK293 cells. Our findings show that the analgesic dose-
dependently inhibits Ca,3.1, Ca,3.2, and Ca,3.3 channel currents. The potency of
eugenol for cloned T-type channel currents isoforms was similar to that for T-type
currents recorded from rat TG neurons. These results strongly suggest that LVA T-type
cannels are potential molecular targets that help account for the dental analgesic’s
ability to relieve pain.
MATERIALS and METHODS

Chemicals

Most of the chemicals used for patch clamp recordings were purchased from Sigma-Aldrich (St. Louis, MO).

HEK293 cell culture and expression of Ca_{3.1}, Ca_{3.2}, and Ca_{3.3} T-type Ca^2+ channel isoforms in HEK293 cells

HEK293 cells that were stably transfected with cDNAs encoding rat Ca_{3.1}, human Ca_{3.2} and rat Ca_{3.3} (Lee et al., 1999) were cultured in DMEM supplemented with 10% FBS containing penicillin and streptomycin (Invitrogen, Carlsbad, CA) at 37°C in a humidified atmosphere of 95% air/5% CO_2. The stable cell lines were prepared in the morning before experiments as follows: cells were dissociated with treatment of 0.05% trypsin-EDTA (Invitrogen) for 2–3 min, then diluted 20-fold with DMEM, after which they were triturated and plated on coverslips coated with 0.1 mg/ml poly-L-lysine (Sigma-Aldrich) and maintained in a humidified CO_2 incubator.

Preparation of TG neurons

TG neurons were isolated from 3- to 4-week old Sprague-Dawley rats (Orient Bio, Inc.,
Gapyeong-City, Korea) using procedures approved by the Animal Care Committee of Sogang University. TG neurons were dissected out of the rat brain and cut into small pieces in Hanks’ balanced salt solution (Invitrogen). TG neurons were incubated in 3 mL Hanks’ balanced salt solution containing 0.25% trypsin (Invitrogen) at 37°C for 30 min and then dissociated by trituration with sterile Pasteur pipettes. Subsequently, TG neurons were plated onto glass coverslips coated with 0.1 mg/ml poly-L-lysine (Sigma-Aldrich) and maintained in a humidified incubator supplied with 95% air/5% CO₂ at 37°C.

**Electrophysiological recording**

Whole-cell patch clamp recordings were performed at room temperature using an Axopatch 200B patch-clamp amplifier (Molecular Devices, Foster City, CA), which was connected to a computer via a Digidata 1322A converter (Molecular Devices, Foster City, CA) and controlled using pCLAMP 9.2 software. Recording pipettes were pulled from TW-150-3 capillary tubing (World Precision Instruments, Sarasota, FL) and then fire polished. When filled with the internal solution, the resistance of the recording electrodes was 2–3 MΩ. The values of series resistance and capacitance were taken directly from readings of the amplifier after electronic subtraction of the capacitive
transient. Series resistance was compensated to 75~80% using the prediction and compensation circuit. Giga seal formation and ruptured patch configuration were achieved in an external solution containing the following (in mM): 140 TEACl, 2.5 CsCl, 10 BaCl₂, 1 MgCl₂, 10 HEPES, 10 glucose, pH = 7.3 with TEAOH. The internal solution contained the following (in mM): 130 CsCl, 10 HEPES, 2 CaCl₂, 10 EGTA, 5 MgATP, and pH = 7.3 with CsOH. Ca₃.1 and Ca₃.2 currents were filtered at 2 kHz and sampled at 10 kHz, while Ca₃.3 currents were filtered at 1 kHz and sampled at 5 kHz. Current amplitudes and exponential fits were obtained using Clampfit 9.2 software. Prism software (GraphPad, San Diego, CA) was used to fit activation and steady-state inactivation data with the Boltzmann equation. The smooth curve for channel activation was obtained by fitting the average data of chord conductance with a Boltzmann equation: \( G = \frac{1}{1 + \exp\left(\frac{V_{50} - V}{S_{\text{act}}}\right)} \), where \( V_{50} \) is the potential for half-maximal activation and \( S_{\text{act}} \) is the slope conductance. The curve for steady-state inactivation was derived from fitting normalized peak amplitudes against voltage with a Boltzmann equation: \( \frac{I}{I_{\text{max}}} = \frac{1}{1 + \exp\left(\frac{V_{50_{\text{inact}}} - V}{k}\right)} \), where \( V_{50_{\text{inact}}} \) is the potential for half-inactivation and \( k \) is the slope factor. Data are presented as mean ± S.E.M. Differences were evaluated for significance using Student’s unpaired \( t \)-tests, with \( P < 0.05 \) (*), \( P < 0.01 \) (**) and \( 0.001 \) (***) as levels of significance.
RESULTS

_Eugenol inhibition of cloned Ca\(_{\text{v}}\)3 T-type channel isoforms_

We first tested the ability of eugenol to inhibit T-type Ca\(^{2+}\) channel isoforms (Ca\(_{\text{v}}\)3.1, Ca\(_{\text{v}}\)3.2, and Ca\(_{\text{v}}\)3.3), each of which was stably transfected in HEK293 cells. Whole-cell patch clamp recordings of T-type Ca\(^{2+}\) channel currents were performed in 10 mM Ba\(^{2+}\) solution as the charge carrier. When serial concentrations of eugenol solutions (100, 300, 1000, and 3000 \(\mu\)M) were cumulatively applied, currents generated through individual T-type isoforms were inhibited in a concentration-dependent manner (right panels of Fig. 1A–C). The time courses of T-type current inhibition by serial eugenol solutions displayed that eugenol inhibition was fast, reaching a steady state within 1 min (left panels of Fig. 1A–C). All the T-type channel currents were strongly inhibited by 3 mM eugenol, more than ~95\%, and the inhibited currents were almost fully recovered within 2 min by washout. Small decrements in current amplitudes after washout were likely due to rundown (<10\%). The dose-response curves were derived from fitting a Hill-Langmuir equation to the data. The IC\(_{50}\) values for eugenol inhibition of Ca\(_{\text{v}}\)3.1, Ca\(_{\text{v}}\)3.2, and Ca\(_{\text{v}}\)3.3 T-type currents were estimated to be 463 ± 12, 486 ± 12, and 708 ± 11 \(\mu\)M, respectively. Statistical analysis shows that Ca\(_{\text{v}}\)3.1 and Ca\(_{\text{v}}\)3.2 were more sensitively inhibited by eugenol than Ca\(_{\text{v}}\)3.3 (Student’s unpaired \(t\)-tests, \(P < 0.05\); \(n=5-13\); Fig. 1D).
**Eugenol effects on current kinetics of T-type Ca,3 channels**

We next examined whether eugenol could alter the current kinetics of Ca,3.1, Ca,3.2, and Ca,3.3 T-type channel currents. When the current traces inhibited by eugenol (1 mM) were normalized to those before eugenol inhibition, the traces generated by Ca,3.1 and Ca,3.2 largely overlapped (Fig. 2A, B), suggesting that eugenol had little effect on the current kinetics of Ca,3.1 and Ca,3.2. We further analyzed Ca,3.1 and Ca,3.2 currents by fitting double exponentials to them, one corresponding to activation and the other to inactivation. No significant differences were detected in the activation and inactivation time constants of Ca,3.1 and Ca,3.2 currents before and after treatment with serial eugenol solutions (Fig. 2D). On the contrary, the normalized Ca,3.3 current traces before and after 1 mM eugenol treatment displayed that the inactivation kinetics of the Ca,3.3 current was accelerated, while the activation kinetics was little altered (Fig. 2C-E). The accelerated inactivation kinetics of the Ca,3.3 current was largely recovered by washout. Additional experiments and data analysis (Fig. 2D) showed that 100 μM eugenol had little effect on the inactivation time constant of Ca,3.3 currents, while 300 μM eugenol slightly decreased the inactivation time constant, which was further decreased by 1 mM eugenol (P < 0.05 and 0.01, Student’s unpaired t-test, n=5-6),
suggesting that the inactivation kinetics of Ca,3.3 currents is accelerated in a concentration-dependent manner. Consistently, analysis of current traces evoked by a current-voltage (I-V) protocol before and after 500 μM eugenol treatment displayed that eugenol accelerated the apparent inactivation kinetics of Ca,3.3 currents at all the test potentials (P < 0.05, 0.01, or 0.001, Student’s unpaired t-tests, n=5-6; Fig. 2E). A likely explanation is slow open channel block during the test pulse, as observed with ethosuximide (Gomora et al., 2001).

Voltage-independent inhibition of Ca,3 T-type channel isoforms by eugenol

Currents from HEK293 cells expressing Ca,3.1, Ca,3.2, or Ca,3.2 channels were elicited by test pulses from a holding potential of -90 mV ranging between -70 mV and +40 mV by 10 mV increments. Representative current traces of Ca,3.1, Ca,3.2, and Ca,3.3 before and after application of 500 μM eugenol are shown for comparison (Fig. 3A-C). Analysis of the I-V relationships (Fig. 3D-F) showed that the eugenol inhibition percentages were similar over all test potentials, suggesting that eugenol inhibition of all T-type channel isoforms is voltage-independent. Consistent with these results, eugenol (500 μM) did not significantly shift the activation curves of the three T-type channel isoforms (Fig. 4).
Negative shifts of steady-state inactivation curves of T-type channels by eugenol

It has been reported that many drugs, including mibefradil, preferentially bind to inactivated state(s) of T-type channels (Martin et al., 2000). We thus examined whether eugenol modified the steady-state inactivation curves of T-type channel isoforms.

Before and after application of 500 μM eugenol, channel availability was measured with a voltage step to -20 mV from varying prepulse potentials from -110 mV to -45 mV (Fig. 4A–C). Smooth curves were obtained from fitting data to a Boltzmann equation, indicating that treatment of 500 μM eugenol negatively shifted the midpoint (V_{50\text{inact}}) of voltage-dependence of inactivation for Cav3.1, Cav3.2, and Cav3.3 by 10.2, 7.4, and 8.6 mV, respectively (P < 0.05, Student’s unpaired t-tests; n=6-8; Fig. 4A–C). The negative shifting effects almost disappeared after washing out the drug (data not shown). The negative shifting effects of channel availability by eugenol suggest that the drug likely bind preferentially to inactivated states of the T-type channel isoforms, thereby shifting the equilibrium away from states from which channels can open (Ertel and Cohen, 1994).

Eugenol binding to the inactivated state of T-type channel isoforms

To evaluate further whether eugenol binds to the inactivated states of T-type channels,
we applied a voltage protocol in which the holding potential was kept at -75 mV to induce ~50% inactivation of the channel. Application of serial concentrations of eugenol inhibited T-type channel currents in a concentration-dependent manner. Analysis of the eugenol inhibition showed that the IC\textsubscript{50} values for Ca\textsubscript{v3.1}, Ca\textsubscript{v3.2}, and Ca\textsubscript{v3.3} at the depolarized holding potential of -75 mV were 192 ± 15, 168 ± 20, and 648 ± 13, respectively (Fig. 5). These results indicate that eugenol blocks of Ca\textsubscript{v3.1} and Ca\textsubscript{v3.2} channel isoforms was enhanced two- to three-fold by the decreased channel availability, while that for Ca\textsubscript{v3.3} was only slightly increased, by 9.2%. These results suggest that the drug preferentially binds to the inactivated states of Ca\textsubscript{v3.1} and Ca\textsubscript{v3.2}.

**Use-dependent enhancement of eugenol inhibition of T-type channel isoforms**

We next examined whether eugenol inhibition of T-type channel isoforms is use-dependent. In Fig. 1, the IC\textsubscript{50} values of eugenol for Ca\textsubscript{v3.1} and Ca\textsubscript{v3.2} were obtained at a stimulation frequency of 0.1 Hz. At an increased stimulation frequency of 0.2 Hz, the inhibition potency by eugenol (500 \(\mu\)M) for Ca\textsubscript{v3.1} and Ca\textsubscript{v3.2} was ~50%, which is similar to the potency at 0.1 Hz. At higher test frequencies of 0.5 and 1 Hz, however, the inhibition percentages of Ca\textsubscript{v3.1} and Ca\textsubscript{v3.2} currents by 500 \(\mu\)M eugenol were significantly enhanced, indicating that eugenol inhibition of T-type channel isoforms is
use-dependent (Fig. 6). One logical interpretation of the use-dependent inhibition profile of eugenol is that the drug preferentially binds to open and inactivated states of T-type channels isoforms.

**Eugenol inhibition profile of T-type currents recorded from TG neurons**

Multiple publications have reported that T-type channels are expressed in TG neurons (Borgland et al., 2001, 2002; Ikeda and Matsumoto, 2003; Ross et al., 2009). To evaluate the sensitivity of eugenol on endogenous T-type currents, we recorded T-type currents from afferent neurons using whole-cell patch clamping. T-type currents displaying transient kinetics were predominantly elicited by a step pulse of -30 (or -40 mV) from a holding potential of -90 mV, but small non-inactivating currents were slightly contaminated in the whole currents. The non-inactivating currents maintained even during the second step pulse, followed by an intervening step potential of -60 mV, are likely to be those passing through HVA calcium channels. Application of serial concentrations of eugenol inhibited T-type currents evoked by step pulses of -30 mV in a concentration-dependent manner. In comparison, non-inactivating HVA currents, which were similarly evoked by the second pulse of -30 mV in amplitude, were less sensitively inhibited by the drug than T-type currents. For example, 1 mM eugenol
inhibited T-type current by 70~80%, but it inhibited HVA current by 40~45%. Analysis of data showed that the IC$_{50}$ value for the TG T-type current is $498.5 \pm 14$ μM (n=3-5). These data indicate that eugenol inhibition sensitivity for TG T-type current is similar to the values for Ca$_v$3.1 and Ca$_v$3.2.

**Nickel sensitive inhibition of T-type currents in TG neurons**

Previous nickel blocking studies of recombinant T-type channel isoforms have established that Ca$_v$3.2 is sensitively blocked by low concentrations of nickel (IC$_{50}$ = 5~12 μM), while Ca$_v$3.1 and Ca$_v$3.3 require much higher concentrations of nickel to be blocked (IC$_{50}$ for Ca$_v$3.1 = 250~305 μM; IC$_{50}$ for Ca$_v$3.3 = 216 μM) (Lee at al., 1999; Kang et al., 2006). Based on the nickel sensitivity, we attempted to evaluate which T-type channel isoforms are mainly expressed in TG neurons. Application of serial Ni$^{2+}$ solutions diminished T-type currents in a concentration-dependent manner and the IC$_{50}$ for nickel blockade was estimated to be $15.2 \pm 1.2$ μM (n=4-6; Fig. 8). These findings suggest that T-type currents in rat TG neurons mainly arise from nickel-sensitive Ca$_v$3.2 isoforms, which may play crucial roles in mediating sensing processes of TG neurons.
DISCUSSION

In this study, we determined the ability of eugenol to inhibit recombinant and endogenous T-type channels, which are potential molecular targets in TG sensory neurons. The IC$_{50}$ values of eugenol for recombinant Ca$_v$3.1, Ca$_v$3.2, and Ca$_v$3.3 channels were 485, 496, and 710 μM, respectively, suggesting that eugenol inhibits Ca$_v$3.1 and Ca$_v$3.2 more sensitively than it does Ca$_v$3.3. When T-type channel currents of TG neurons were recorded, the TG T-type currents showed transient kinetics that were more similar to the fast kinetics of Ca$_v$3.1 and Ca$_v$3.2 currents than to the slow kinetics of Ca$_v$3.3. Accordingly, the inhibition profile of eugenol for native T-type currents showed that the eugenol inhibition sensitivity for native T-type currents is similar to that for Ca$_v$3.1 and Ca$_v$3.2 rather than Ca$_v$3.3.

LVA T-type Ca$^{2+}$ channels are known to be activated at lower threshold potential than voltage-gated Na$^+$ channels and HVA Ca$^{2+}$ channels (McCormick and Huguenard, 1992; Jahnsen and Llinás, 1984; Llinás and Steriade, 2006). In thalamic neurons, for example, a burst of action potentials can be triggered by low-threshold calcium spikes mediated by T-type Ca$^{2+}$ channels. Inhibition of the calcium spikes by divalent metal ions can prevent triggering TTX-sensitive burst firing (Jahnsen and Llinás, 1984). Based on the previous reports addressing lower activation threshold of T-
type channels, our findings that eugenol inhibits LVA Ca\textsubscript{v}3.1 and Ca\textsubscript{v}3.2 isoforms more sensitively than HVA Ca\textsuperscript{2+} channels and Na\textsuperscript{+} channels strongly suggest that LVA Ca\textsuperscript{2+} channels are more critical target proteins than Na\textsuperscript{+} and HVA Ca\textsuperscript{2+} channels for the pain-relieving effects of eugenol.

We showed that the eugenol inhibition sensitivity for Ca\textsubscript{v}3.1 and Ca\textsubscript{v}3.2 was enhanced 2-3 fold by a change of holding potential from -90 mV to -75 mV to induce ~50\% inactivation of the channel (Fig. 5). In native TG neurons, T-type channels seem to be inactivated by more than ~50\%, because the resting membrane potential of isolated TG neurons was previously found to be around -63 mV (Martenson et al., 1997). We interpret that the eugenol sensitivity for Ca\textsubscript{v}3.1 and Ca\textsubscript{v}3.2 would be further enhanced by the depolarized conditions of TG neurons, thereby eugenol block of T-type currents contributing to eugenol’s analgesic mechanism of action.

Comparison of biophysical properties of T-type channel isoforms before and after eugenol treatment demonstrated that eugenol negatively shifts the steady-state inactivation curves of three T-type channel isoforms in common. The negative shifting effects of eugenol significantly reduced the window currents, as illustrated by overlapping of the activation and steady-state inactivation curves (Fig. 3A-C). This would provide a second mechanism by which eugenol decreases the excitability of TG neurons.
sensory neurons, and contributes to its ability to relieve pain during dental treatment.

Due to its alleviation action of tooth pain, eugenol has been widely utilized as a local anesthetic agent in human dentistry (Markowitz et al., 1992). In rat models, the agent has been demonstrated to be effective in relieving pain from orofacial regions and other peripheral regions (Park et al., 2009; Lionnet et al., 2010; Yeon et al., 2011). For example, Park and his colleagues showed that subcutaneous injection of eugenol into the left vibrissa pad dose-dependently decreased the latency time of head withdrawal in response to thermal stimuli and application of eugenol onto inferior alveolar nerve reduced jaw-opening reflex in response to noxious electrical stimulation to anterioal tooth pulp. They suggested that eugenol inhibited the formation of action potentials and the nerve conduction of sensory signals via blocking of diverse ion channels including voltage-gated Na$^+$ channels in TG neurons (Park et al., 2009).

We here investigated the eugenol inhibition profiles for both cloned and endogenous T-type channels in TG neurons with their IC$_{50}$ values of ~500 μM. Eugenol has been reported to inhibit multiple ion channels detected in TG neurons as follows: the IC$_{50}$ values for voltage-activated Na$^+$ channels, HVA Ca$^{2+}$ channels, voltage-activated K$^+$ channels, and hyperpolarization-activated cation channels are ~ 600 μM, 1 mM, 376 μM, and 157 μM, respectively (Lee et al., 2005; Park et al., 2006 and 2009; Li
et al., 2007; Yeon et al., 2011). Taken together with the previous findings, we suggest that T-type channels are additional molecular targets for eugenol and therefore are likely to play a part in eugenol’s underlying mechanism of analgesia.
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AUTHORSHIP CONTRIBUTIONS

- Participated in research design: Seo, Li, and Lee

- Conducted experiments: Seo, Lee

- Performed data analysis: Seo, Perez-Ryes, and Lee

- Wrote the manuscript: Perez-Reyes and Lee
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**FOOTNOTES**

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LEGENDS FOR FIGURES

Figure 1. Eugenol inhibition of Ca\textsubscript{v}3.1, Ca\textsubscript{v}3.2, and Ca\textsubscript{v}3.3 T-type Ca\textsuperscript{2+} isoforms. A–C. Time courses of the inhibitory effects of eugenol (0.1, 0.3, 1, and 3 mM) on T-type channel currents from HEK293 cells stably transfected with Ca\textsubscript{v}3.1 (A), Ca\textsubscript{v}3.2 (B), or Ca\textsubscript{v}3.3 (C). Currents were elicited by a test pulse of -20 mV from a holding potential of -90 mV every 10 sec. Their representative current traces before and after eugenol inhibition are superimposed, as shown on the right side of each time course. D. Dose-response curves of eugenol inhibition of T-type channel isoforms. Inhibition percentages of Ca\textsubscript{v}3.1, Ca\textsubscript{v}3.2, and Ca\textsubscript{v}3.3 channel currents by serial concentrations of eugenol were averaged and plotted against eugenol concentrations (n=5–13). The smooth curves were obtained from fitting data with the Hill equation, I = \frac{(1 + IC_{50}/(\text{eugenol})^n)^{-1}}{1 + IC_{50}/(\text{eugenol})^n}, where I is the normalized inhibition, \text{IC}_{50} is the concentration of eugenol required for half maximal inhibition, and n is the Hill coefficient.

Figure 2. Eugenol effects on the current kinetics of T-type channel isoforms. A-C. Ca\textsubscript{v}3.1 (A), Ca\textsubscript{v}3.2 (B), and Ca\textsubscript{v}3.3 (C) currents were elicited by step pulses to -20 mV from a holding potential of -90 mV. Representative traces before (black) and after application of 1 mM eugenol (grey), and after washout (dashed) of the drug are...
superimposed and are shown in the upper panels. The current traces were normalized to their peak current amplitude before eugenol treatment (see lower panels of Fig. 2A-C), indicating that the inactivation rate of only the Ca_3.3 current was accelerated by eugenol inhibition. **D. Eugenol effects on the inactivation time constants (τ_{inact}) of Ca_3.1, Ca_3.2, and Ca_3.3 currents.** The inactivation portion of each current was fitted with a single exponential equation (I = A*exp(-t/τ) + B), and the average value inactivation time constants (τ) are illustrated with bar graphs. Eugenol only accelerated the inactivation kinetics for Ca_3.3 in a concentration-dependent manner (p < 0.05 (*) or 0.01 (**), Student’s t-test; n=5-6). **E. Eugenol effects on the inactivation time constants of Ca_3.3 currents evoked by various test potentials.** Currents elicited by serial step pulses ranging from -70 mV to +40 mV from a holding potential of -90 mV were measured before and after treatment with application of 500 μM. Currents were fitted with two exponential equations (I = A_1 exp(-t/τ_1) + A_2 exp(-t/τ_2) + C), where τ_1 is the inactivation time constant and τ_2 is the activation time constant. Average inactivation tau (τ) values before (○) and after 500 μM eugenol treatment (●) are plotted as a function of test potential. Levels of significance (P < 0.05, P < 0.01, or 0.001) evaluated by Student’s unpaired t-tests are marked with asterisks (*, **, or ***), respectively (n=5-6). Data represent the mean ± S.E.M.
Figure 3. Eugenol effects on the current-voltage (I-V) relationships of T-type channel isoforms. A-C. Representative current traces of Ca_{v}3.1 (A), Ca_{v}3.2 (B) and Ca_{v}3.3 (C) before and after application of 500 μM eugenol were elicited by a voltage protocol composed of serial step pulses ranging from -70 mV to +40 mV by increments of 10 mV from a holding potential of -90 mV. D–F. I-V relationships of Ca_{v}3.1, Ca_{v}3.2, and Ca_{v}3.3 channels before and after 500 μM eugenol treatment. Currents measured at various test potentials were normalized to the peak current at a test potential of -20 mV, and their normalized percentages are plotted against test potentials applied (n=5-9). Eugenol inhibition percentages over different potentials are similar, suggesting that eugenol inhibition of T-type channel isoforms is voltage-independent.

Figure 4. Eugenol effects on the activation and channel availability curves of T-type channels. Activation and steady-state inactivation curves of Ca_{v}3.1 (A, circles), Ca_{v}3.2 (B, triangles), and Ca_{v}3.3 (C, squares) are displayed from before (open symbols) and after 500 μM eugenol treatment (closed symbols). Channel activation levels depending on voltage are from the chord conductance values obtained by dividing current amplitudes by driving forces (reversal potential minus test potential), normalized to the peak conductance. The normalized chord conductance values were
averaged and plotted against the test potentials. Smooth curves were obtained from fitting the data to the Boltzmann equation \( G = \frac{1}{1+\exp(V_{50,\text{act}}-V)/k} \), where \( V_{50,\text{act}} \) is the half-activation voltage and \( k \) is a slope factor. The steady-state inactivation was evaluated by a two-step pulse protocol: A conditioning pulse of 10 sec duration ranging from -110 mV to -45 mV by increments of 5 or 10 mV was followed by a step to -20 mV test potential. Currents recorded at -20 mV after the serial conditioning potentials were normalized to the current amplitude recorded at a conditioning potential of -90 mV, and then average inactivation percentages were plotted as a function of prepulse potentials. The normalized data were then fit to the Boltzmann equation. Data represent the mean ± S.E.M (n=6-8).

Figure 5. State-dependent inhibition effects of eugenol on T-type channel isoforms. Ca_{v}3.1 (A), Ca_{v}3.2 (C), and Ca_{v}3.3 (E) T-type currents were evoked by a test potential of -20 mV from different holding potentials of -90 mV (upper panels) or -75 mV (lower panels) in which the latter decreases channel availability to ~50%. Representative current traces before and after serial concentrations of eugenol are superimposed and shown on the left side. B, D, and F. Dose-response curves of eugenol inhibition of T-type channel isoform currents. Inhibition percentages of Ca_{v}3.1 (B), Ca_{v}3.2 (D), and
Ca_{3.3} (F) currents evoked from a holding potential of -90 mV (open symbols) or -75 mV (closed symbols) were averaged and plotted against eugenol concentrations (n=5–13). The smooth curves are obtained from fitting data to the Hill equation, I = \left \{ 1 + \frac{IC_{50}}{\text{eugenol}^n} \right \}^{-1}, where I is the normalized inhibition, IC_{50} is the concentration of eugenol required for half maximal inhibition, and n is the Hill coefficient.

**Figure 6. Use-dependent inhibition of T-type channel currents by eugenol.**

Ca_{\text{V}3.1} (A), Ca_{\text{V}3.2} (B), and Ca_{\text{V}3.3} (C) currents were elicited by a step pulse of -20 mV from a holding potential of -90 mV with various frequencies of 0.2 Hz (□), 0.5 Hz (△), or 1 Hz (○). After current amplitude was stabilized, 500 μM eugenol was applied. The peak amplitudes of currents were normalized to the peak current amplitude before application of eugenol, and then the normalized average values (mean ± SEM) were plotted against time (n=4-5).

**Figure 7. Eugenol inhibition profile of T-type currents recorded from TG neurons.**

A. Ca^{2+} channel currents recorded in a 10 mM Ba^{2+} solution are superimposed before and after application of serial eugenol solutions (0.1, 0.3, and 1 mM). Currents were elicited every 10 sec by a double pulse protocol composed of double step pulses of -30
mV and a intervening potential of -60 mV. **B.** Isolation of pure T-type channel currents by subtracting the data points during the “b” period from those during “a” period. T-type channel currents isolated are superimposed before and after treatment of eugenol solutions (0.1, 0.3, and 1 mM). **C.** Concentration-response curves of eugenol inhibition of T-type channel currents recorded from TG neurons. Inhibition percentages of TG neuron T-type currents by serial concentrations of eugenol were averaged and plotted against eugenol concentrations (n=3–5). The smooth curves were obtained from fitting data to the Hill equation, \( I = \frac{1 + IC_{50}/(\text{eugenol})^n}{1} \), where \( I \) is the normalized inhibition, \( IC_{50} \) is the concentration of eugenol required for half maximal inhibition, and \( n \) is the Hill coefficient.

**Figure 8. Nickel sensitive inhibition of T-type currents recorded from TG neurons.**

**A.** Representative T-type current traces before and after serial nickel solutions (1, 3, 10, and 30 \( \mu \)M) are superimposed. T-type channels currents from rat trigeminal ganglion (TG) neurons were elicited by a step pulse of -35 (or -40) mV every 10 sec. **B.** Concentration-response curve of nickel inhibition of T-type channel currents recorded from TG neurons. Inhibition percentages of T-type channel currents by serial concentrations of nickel were averaged and plotted against nickel concentrations (n=4-
6). The smooth curve is obtained from fitting data with the Hill equation, \( I = \left\{ 1 + \frac{\text{IC}_{50}}{\text{IC}_{50}^{2+y^2}} \right\}^{-1} \), where I is the normalized inhibition, \( \text{IC}_{50} \) is the concentration of nickel required for half maximal inhibition, and \( n \) is the Hill coefficient.
Figure 1

A. $\text{CaV}_3.1$

B. $\text{CaV}_3.2$

C. $\text{CaV}_3.3$

D. Percent inhibition (%)

- $\text{CaV}_3.1$ (463)
- $\text{CaV}_3.2$ (486)
- $\text{CaV}_3.3$ (708)

$\text{[Eugenol]}, \mu M$

Time (sec)

I/Imax (%)
Figure 2

A. Ca$_{\text{V}}$3.1

B. Ca$_{\text{V}}$3.2

C. Ca$_{\text{V}}$3.3

D. [Eugenol] (μM)

E. Test Potential (mV)

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Figure 3

A. **Ca\textsubscript{v}3.1**

B. **Ca\textsubscript{v}3.2**

C. **Ca\textsubscript{v}3.3**

D. Test Potential (mV)

E. Test Potential (mV)

F. Test Potential (mV)

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Figure 4

A. $\text{Ca}_\text{v}3.1$

B. $\text{Ca}_\text{v}3.2$

C. $\text{Ca}_\text{v}3.3$
Figure 5

A.  
B.  
C.  
D.  
E.  
F.
Figure 6

A. CaV3.1

B. CaV3.2

C. CaV3.3

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Figure 7

A. [Graph showing current voltage relationships with various concentrations of a compound.]

B. [Graph showing current voltage relationships with the label 'T-type only (a-b).']

C. [Graph showing percent inhibition with [Eugenol] concentration. IC50 = 499 μM]
Figure 8

A.

-35 mV
-90 mV

Nickel
30 μM
10 μM
3 μM
1 μM

control

200 pA
50 ms

B.

Percent inhibition

IC₅₀ = 15.2 μM

[Nickel], μM