# Eugenol Inhibits Calcium Currents in Dental Afferent Neurons

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M.H. Lee†, K.-Y. Yeon†, C.-K. Park, H.-Y. Li, Z. Fang, M.S. Kim, S.-Y. Choi, S.J. Lee, S. Lee[2], K. Park, J.-H. Lee, J.S. Kim, and S.B. Oh[1]*

[1]Department of Physiology, and [2]Department of Craniomaxillofacial Cell and Developmental Biology, College of Dentistry and Dental Research Institute, Seoul National University, 28-2 Yeongeon-Dong Chongno-Ku Seoul, Seoul 110-749, Korea; †authors contributing equally to this work; *corresponding author, odolbae@snu.ac.kr

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## ABSTRACT

Eugenol is a topical analgesic agent widely used in the dental clinic. To elucidate the molecular mechanism underlying its analgesic action, we investigated the effect of eugenol on high-voltage-activated calcium channel (HVACC) currents in dental primary afferent neurons, and with a heterologous expression system. Dental primary afferent neurons were identified by retrograde labeling with a fluorescent dye, DiI. Eugenol inhibited HVACC currents in both capsaicin-sensitive and capsaicin-insensitive dental primary afferent neurons. The HVACC inhibition by eugenol was not blocked by capsazepine, a competitive transient receptor potential vanilloid 1 (TRPV1) antagonist. Eugenol inhibited N-type calcium currents in the cell line C2D7, stably expressing the human N-type calcium channels, where TRPV1 was not endogenously expressed. Our results suggest that the HVACC inhibition by eugenol in dental primary afferent neurons, which is not mediated by TRPV1 activation, might contribute to eugenol's analgesic effect. Abbreviations: high-voltage-activated calcium channel, HVACC; transient receptor potential vanilloid 1, TRPV1; trigeminal ganglion, TG; dorsal root ganglion, DRG; capsazepine, CZP.

**KEY WORDS:** eugenol, transient receptor potential vanilloid 1, trigeminal ganglion, C2D7 cells, high-voltage-activated calcium currents.

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## INTRODUCTION

Eugenol has been widely used in dental clinics to relieve pain (Markowitz *et al.*, 1992). Recently, we have demonstrated that the influx of cations through the activation of transient receptor potential vanilloid 1 (TRPV1), thereby inducing depolarization of the membrane potential to evoke action potentials, could be one of the major molecular mechanisms by which eugenol produces irritant actions (Yang *et al.*, 2003). However, because response patterns of eugenol were distinct from those of capsaicin, we suggested that TRPV1-independent mechanisms also appear to be involved in the pharmacological actions of eugenol (Yang *et al.*, 2003).

In spite of its widespread therapeutic use as a topical analgesic in dentistry, little is known about the mechanisms underlying the analgesic properties of eugenol. It has been reported that beneficial effects of eugenol might be due to inhibition of the cyclo-oxygenase enzyme (Thompson and Eling, 1989; Dohi *et al.*, 1991). However, the inhibitory effect of eugenol on nerve transmission (Trowbridge *et al.*, 1982; Brodin and Roed, 1984) implies its potential actions on ion channels. However, to date, it remains unclear whether eugenol regulates ion channels in sensory neurons, including dental primary afferent neurons.

Voltage-activated calcium channels are key mediators in sensory neurons and play a crucial role in the transmission of pain signals at the spinal/medullary dorsal horn level (Altier and Zamponi, 2004). Capsaicin has been demonstrated to inhibit high-voltage-activated calcium channel (HVACC) currents (Petersen *et al.*, 1989; Bleakman *et al.*, 1990). Since the chemical structure of eugenol is similar to that of capsaicin (Sterner and Szallasi, 1999; Szallasi and Blumberg, 1999), there is a possibility that eugenol modulates HVACC as capsaicin does in sensory neurons (Petersen *et al.*, 1989; Bleakman *et al.*, 1990). In the present study, we investigated if eugenol would regulate HVACC in rat dental primary afferent neurons, and with a heterologous expression system.

## MATERIALS & METHODS

All procedures for animal use were reviewed and approved by the Animal Care and Use Committee of the Seoul National University prior to the experiments.

### Labeling of Dental Primary Afferent Neurons and Cell Preparation

The dental primary afferent neurons were identified by retrograde labeling with a fluorescent dye, DiI (D-282, Molecular Probes, Eugene, OR, USA), as done previously by Chaudhary *et al.* (2001). Trigeminal ganglion (TG) neurons from adult rats (n = 50) were prepared as previously described (Yang *et al.*, 2003). We used C2D7 cells stably expressing the human N-type calcium channel, encoded by the subunits $\alpha_{1B\text{-}a}$, $\beta_{1b}$, $\alpha_{2b}\delta$ (McNaughton *et al.*, 1998), as a heterologous expression system. TRPV1 generated in our lab (Yang *et al.*, 2003) was transiently transfected to C2D7 cells.

### mRNA Preparation and Reverse-transcription/Polymerase Chain-reaction (RT-PCR)

Total RNA was prepared from C2D7 cells and adult TG/DRG neurons with the use of Trizol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. First-strand cDNA was synthesized with the Superscript™ Preamplification System (Invitrogen). PCR reaction was performed with 2 μL of the resulting cDNA, with the use of Taq DNA polymerase (Invitrogen), and primers for PCR were specifically designed for TRPV1 based on GenBank rat cDNA sequences. The primers (forward/reverse) used for the amplification of TRPV1 were CAGACAGAGACCCTAACTGC/CTGTCTGGCCCTTGTAGTA G. PCR reactions with both cDNA from neonatal rat TG/DRG neurons and water were run in parallel as positive and negative controls, respectively.

### Electrophysiological Recordings

We performed whole-cell patch-clamp recordings to measure Ba currents ($I_{Ba}$) with an Axopatch-1C amplifier (Axon Instruments, Union City, CA, USA). The pipette resistance was 2-5 MΩ. Series resistance was compensated for (> 80%), and leak subtraction was performed. Data were low-pass-filtered at 2 kHz, and sampled at 10 kHz. The pClamp8 (Axon Instruments) software was used during experiments and analysis. The pipette solution for $I_{Ba}$ was composed of (mM): CsCl 100, $MgCl_2$ 1, HEPES 10, BAPTA 10, Mg-ATP 3.6, phosphocreatine 14, GTP 0.1, and creatine phosphokinase 50 units/mL, adjusted to pH 7.4 with CsOH. The extracellular solution for $I_{Ba}$ contained (mM): tetraethylammonium chloride (TEACl) 151, HEPES 10, $BaCl_2$ 5, $MgCl_2$ 1, and glucose 10, adjusted to pH 7.4 with TEAOH. The $I_{Ba}$ was evoked by a test pulse to +0 mV from the holding potential, -80 mV every 10 sec.

### Drugs

Eugenol, capsaicin, and capsazepine, purchased from Sigma, were dissolved in dimethylsulfoxide (DMSO) to make stock solutions, and were kept at -20°C. The final concentration of DMSO was less than 0.1% (v/v), which did not affect membrane currents. The drugs were diluted to their final concentration in the extracellular solution, and then applied by gravity through a bath perfusion system. Most neurons were exposed to only a single dosage of eugenol, and the results were averaged across neurons. Because the extents of $I_{Ba}$ inhibition by eugenol were not different even after the exposure to capsaicin (data not shown), and our previous study demonstrated no cross-desensitization between the responses of eugenol and those of capsaicin (Yang *et al.*, 2003), the sensitivity of eugenol-responsive cells to capsaicin was determined based on the $I_{Ba}$ inhibition by 1 μM capsaicin, either before or after the exposure of cells to eugenol. The bath solution was continuous during the experiment at a rate of 1 mL/min.

### Statistical Analysis

Data are expressed as mean ± SEM. ANOVA or unpaired Student's *t* test, along with the software Origin 6.0, was used to determine differences. Differences were considered to be significant when the *P* value was less than 0.05.

## RESULTS

### Eugenol Inhibited HVACC Currents in Dental Primary Afferent Neurons

When the acutely isolated TG neurons were visualized under fluorescent microscopy after 3 wks of DiI crystal placement into both sides of maxillary molar, DiI-labeled neurons, ~ 40 to 60 TG neurons *per* animal, were clearly detectable (Fig. 1A). Using these dental primary afferent neurons, we examined whether eugenol would regulate HVACC currents. Whole-cell recordings of $Ca^{2+}$ currents, with 5 mM $Ba^{2+}$ as the charge carrier ($I_{Ba}$), were performed under experimental conditions that suppressed other voltage-dependent currents, such as $Na^+$ and $K^+$ currents. Membrane currents did not change during the application of 1 mM eugenol (n = 13) (Fig. 1B). However, when dental primary afferent neurons, which ranged from 15 to 50 μm in diameter, were exposed to eugenol (1 mM), the peak $I_{Ba}$ was readily inhibited in a reversible manner (Fig. 1C). In only 12% of neurons tested (n = 5/43) was the $I_{Ba}$ inhibition not recovered to the basal level, and these data were excluded from the results.

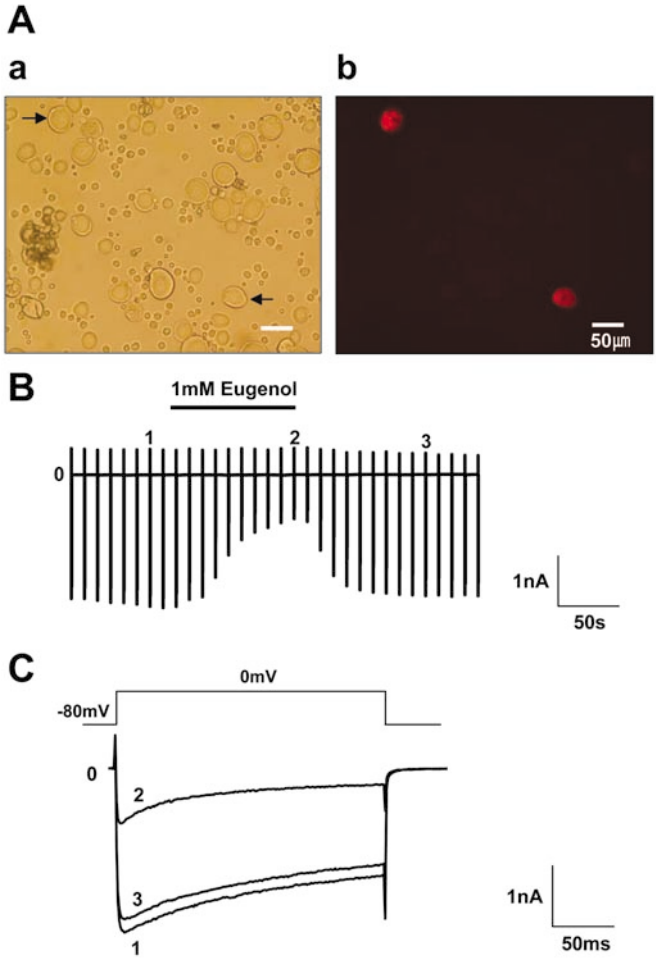


**Figure 1.** Inhibition of HVACC currents by eugenol in dental primary afferent neurons. **(Aa)** Trigeminal neurons were acutely isolated from adult rats 3 wks after placement of a fluorescent dye, DiI, into maxillary molars. Arrows indicate the 2 labeled cells when visualized under fluorescence microscopy **(Ab).** Scale bar, 50 μm. **(B)** The effect of 1 mM eugenol on membrane currents and HVACC barium currents ($I_{Ba}$). B shows continuous chart records of membrane current with eugenol applied at the time indicated by the horizontal bar (1, before; 2, during; 3, after the application of eugenol). Eugenol produced inhibitory effects on $I_{Ba}$ without the change in membrane-holding currents (n = 13). **(C)** Superimposed $I_{Ba}$ evoked by test pulse at the points indicated in (B). Individual currents are numbered on the chart record for ease of identification and superimposed at higher time resolution in (B). Calcium currents were recorded during 200-ms voltage clamp steps from a holding potential of -80 mV to a test potential of 0 mV every 10 sec.

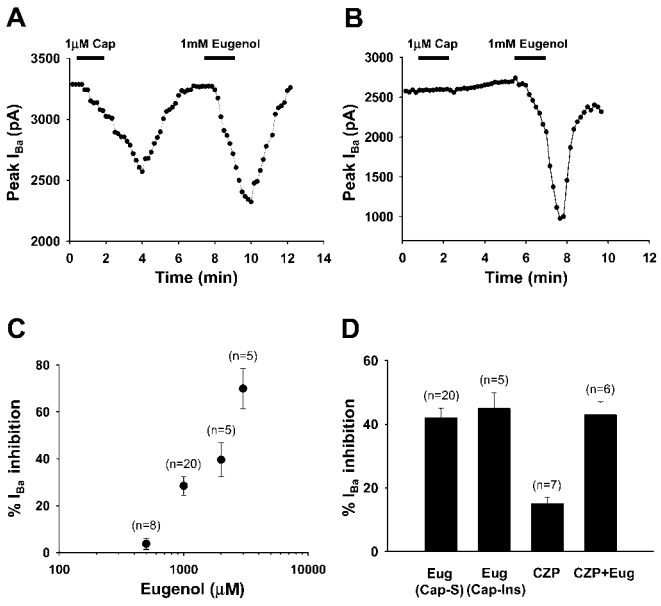


**Figure 2.** Effect of eugenol on $I_{Ba}$ in capsaicin-sensitive and capsaicin-insensitive neurons. **(A)** Time course of the effect of capsaicin (1 μM) and eugenol (1 mM) on $I_{Ba}$. The $I_{Ba}$ inhibition by eugenol (1 mM) was observed in capsaicin-sensitive dental primary afferent neurons (n = 20). **(B)** In dental primary afferent neurons in which $I_{Ba}$ was not inhibited by capsaicin (1 mM), eugenol (1 mM) also inhibited $I_{Ba}$, indicating that the inhibitory effects of eugenol on $I_{Ba}$ can be induced without the involvement of TRPV1 activation. **(C)** Dose-response relationship of eugenol-induced $I_{Ba}$ inhibition in capsaicin-sensitive dental primary afferent neurons (mean ± SEM). The numbers in parentheses represent the numbers of cells studied. **(D)** The summary of $I_{Ba}$ inhibition in dental primary afferent neurons. 1 mM eugenol (Eug)-induced $I_{Ba}$ inhibition in capsaicin-insensitive neurons (Cap-Ins) was similar to that obtained in capsaicin-sensitive neurons (Cap-S). Capsazepine (CZP, 10 μM) failed to block eugenol (1 mM)-induced $I_{Ba}$ inhibition completely. The $I_{Ba}$ inhibition by the combined application of eugenol and CZP was not significantly different from that of eugenol (mean ± SEM, $p > 0.05$).

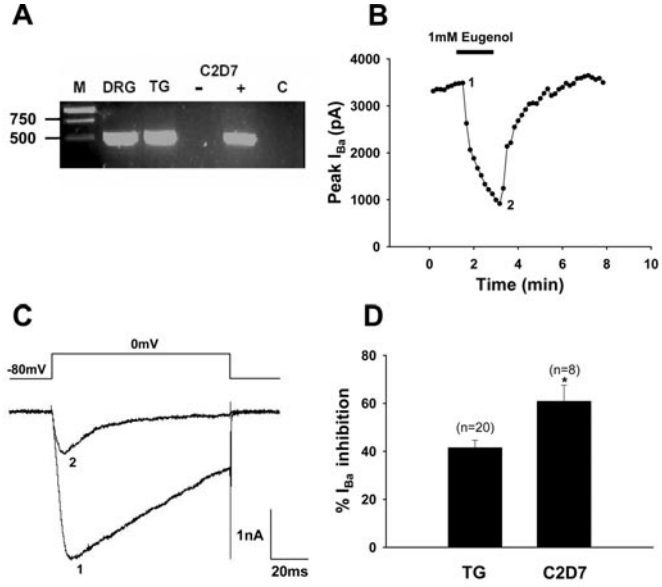


**Figure 3.** Inhibition of N-type Ca currents by eugenol in C2D7 cells. **(A)** RT-PCR analysis shows no endogenous expression of TRPV1 in C2D7 cells (lane -). TRPV1 is clearly expressed in C2D7 cells following transient transfection (lane +), and in both DRG and TG neurons. The expected size of PCR product was 536 bp. Lane C indicates no PCR products with $H_2O$. **(B)** Time course of the effect of eugenol (1 mM) on $I_{Ba}$ in C2D7 cells which have no endogenous TRPV1 expression (n = 5). **(C)** Superimposed $I_{Ba}$ evoked by a test pulse at the points indicated in (B). **(D)** The summary of $I_{Ba}$ inhibition in TG neurons and C2D7 cells. The $I_{Ba}$ inhibition by eugenol in C2D7 cells was significantly greater than that in TG neurons (mean ± SEM, *$p < 0.01$). The number in parentheses represents the number of cells studied.

## Eugenol Inhibited Ca Currents in Both Capsaicin-sensitive and Capsaicin-insensitive Dental Primary Afferent Neurons

We next explored whether the inhibitory effect of eugenol on HVACC would be specific only in capsaicin-sensitive dental primary afferent neurons. We found that eugenol inhibited $I_{Ba}$ in both capsaicin-sensitive neurons and capsaicin-insensitive neurons (Figs. 2A, 2B). Moreover, eugenol also inhibited $I_{Ba}$ in other TG neurons, as well as dental primary afferent neurons (data not shown). The magnitude of $I_{Ba}$ inhibition by 1 mM eugenol in capsaicin-insensitive dental primary afferent neurons (45 ± 5%) (n = 5) (Fig. 2B) was similar to that obtained in capsaicin-sensitive dental primary afferent neurons (42 ± 3%, n = 20) (Figs. 2A, 2D). The inhibition of the peak $I_{Ba}$ in capsaicin-sensitive dental primary afferent neurons was dose-dependent (Fig. 2C). To determine whether the inhibitory effect of eugenol on HVACC was mediated by TRPV1, we examined the effects of eugenol in the presence of 10 μM capsazepine (CZP) (Chaudhary *et al.*, 2001). When we applied 10 μM CZP, a competitive TRPV1 antagonist, for 5 min, it produced inhibitory effects on HVACC (n = 7) (Fig. 2D), as previously reported (Docherty *et al.*, 1997). However, the extent of TRPV1-independent CZP-induced $I_{Ba}$ inhibition (15 ± 2%) was smaller than that in DRG neurons (Docherty *et al.*, 1997). The application of 10 μM CZP together with 1 mM eugenol inhibited $I_{Ba}$ by 43 ± 4% (n = 6), which was not significantly different from the inhibition by eugenol alone (42 ± 3%) (n = 20) (Fig. 2D).

## Inhibition of Ca Currents by Eugenol Does Not Require VR1 Activation

We then tested if the $I_{Ba}$ inhibition by eugenol was mediated by the activation of TRPV1 in a heterologous system—a cell line, C2D7, that stably expresses human N-type calcium channels. RT-PCR analysis indicated that TRPV1 was not endogenously expressed in C2D7 cells, whereas DRG and TG expressed TRPV1 (Fig. 3A). In these C2D7 cells (n = 5), 1 mM eugenol still produced an inhibitory effect on N-type calcium currents (Figs. 3B, 3C). Interestingly, the $I_{Ba}$ inhibition was significantly bigger than that in TG neurons (Fig. 3D).

# DISCUSSION

The analgesic pharmacological action elicited by eugenol is the basis for its wide use in dental clinics as a sedative and anodyne agent. In the present study, we investigated whether eugenol would modulate HVACC currents. We found that eugenol elicited an inhibitory effect on HVACC in all TG neurons, including dental primary afferent neurons (Fig. 1), and these effects were not restricted to only capsaicin-sensitive dental primary afferent neurons (Fig. 2). Analysis of these findings implies that, although eugenol and capsaicin share

vanillyl-like moiety in their chemical structures (Sterner and Szallasi, 1999; Szallasi and Blumberg, 1999), the mechanisms underlying inhibitory effects between eugenol and capsaicin on HVACC might be different. Capsaicin reduces HVACC currents of rat DRG neurons through the mediation of capsaicin receptor activation (Bleakman *et al.*, 1990), which indicates the involvement of TRPV1 in the inhibitory effects of capsaicin on calcium channels. We also found, using TG neurons and a heterologous expression system, that TRPV1 activation is required for the inhibition of calcium current by capsaicin (unpublished data). Moreover, CZP, a competitive TRPV1 antagonist, failed to block the inhibitory effects of eugenol on HVACC completely (Fig. 2D). The extent of HVACC inhibition by eugenol was comparable with the combined application of eugenol and CZP, showing that the inhibitory effect on HVACC produced by eugenol was not mediated by TRPV1 activation (Fig. 2D). Taken together, the activation of TRPV1 in dental primary afferent neurons is not likely to be involved in the eugenol-induced inhibitory effects on HVACC.

TRPV1-independency to eugenol action was further confirmed with a heterologous system. In C2D7 cells that do not contain endogenous TRPV1 (Fig. 3A), eugenol produced inhibitory effects on the N-type calcium channel, the major subtype of HVACC in sensory neurons (Wilson *et al.*, 2000), clearly demonstrating that the involvement of TRPV1 is not a prerequisite for eugenol-induced inhibitory effects on calcium channels. It was interesting to note that inhibitory effects of eugenol on N-type currents in C2D7 cells were significantly stronger than those on HVACC in TG neurons. Because HVACC currents result from diverse subtypes of calcium channels expressed in sensory neurons (Wilson *et al.*, 2000), it is likely that inhibitory effects of eugenol are preferentially stronger on N-type calcium channels than on other subtypes of calcium channels (Fig. 3D).

The concentration of eugenol in the cavity preparation below ZOE has been reported to be $10^{-2}$ ~ $10^{-4}$ M (Craig and Powers, 2002). This supports our contention that the range of eugenol concentration (~ $10^{-3}$ to $10^{-4}$ M), which produced inhibitory effects on HVACC in our study, corresponds to that used in dental therapeutic applications. Our findings that the inhibitory effects of eugenol on HVACC currents were readily reversible and dose-dependent argue against the inhibitory effects on Ca channels being due to the non-specific effects by rather high concentrations of eugenol. The inhibition of prostaglandin and leukotriene synthesis may also contribute to the analgesic effects of eugenol (Hirafuji, 1984; Dohi *et al.*, 1991).

It has been demonstrated that eugenol given intrathecally produced anti-nociceptive effects *via* the capsaicin receptors located on sensory terminals in the spinal cord (Ohkubo and Shibata, 1997). Although our data are not consistent with this report in terms of TRPV1 involvement, the inhibition of HVACC—thereby decreasing Ca-dependent neurotransmitter release from the pre-synaptic terminals of the neurons in the medullary dorsal horn—could be one of the mechanisms by which eugenol produces its analgesic effects.

In summary, we demonstrate that eugenol produces inhibitory effects on HVACC currents in dental primary afferent neurons, and these inhibitory actions could contribute to the analgesic effect produced by eugenol. However, TRPV1 activation is not a prerequisite for the inhibitory effects of eugenol on HVACC.

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