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Eugenol Inhibits Sodium Currents in Dental Afferent Neurons

ABSTRACT

Although eugenol is widely used in dentistry, little is known about the molecular mechanisms responsible for its anesthetic properties. In addition to calcium channels, recently demonstrated by our group, there could be another molecular target for eugenol. Using a whole-cell patch-clamp technique, we investigated the effect of eugenol on voltage-gated sodium channel currents (I_{Na}) in rat dental primary afferent neurons identified by retrograde labeling with a fluorescent dye in maxillary molars. Eugenol inhibited action potentials and I_{Na} in both capsaicin-sensitive and capsaicin-insensitive neurons. The pre-treatment with capsazepine, a competitive antagonist of transient receptor potential vanilloid 1 (TRPV1), failed to block the inhibitory effect of eugenol on I_{Na}, suggesting no involvement of TRPV1. Two types of I_{Na}, tetrodotoxin (TTX)-resistant and TTX-sensitive I_{Na}, were inhibited by eugenol. Our results demonstrated that eugenol inhibits I_{Na} in a TRPV1-independent manner. We suggest that I_{Na} inhibition by eugenol contributes to its analgesic effect.

KEY WORDS: eugenol, trigeminal ganglion neurons, voltage-gated sodium channels.

INTRODUCTION

Eugenol is extensively used in dentistry, due to its ability to allay tooth pain. However, little is known about the molecular mechanisms underlying its anesthetic properties. It has been shown that eugenol irreversibly blocked the conduction of action potential in the frog sciatic nerve (Kozam, 1977). Eugenol slowed nerve conduction in crayfish neurons and made them less excitable (Ozeki, 1975). Eugenol inhibited intradental nerve activity in cats (Trowbridge *et al.*, 1982), as well as compound action potentials in the rat phrenic nerve in a reversible manner, like local anesthetics (Brodin and Roed, 1984). All of these *in vivo* and *ex vivo* results suggest that eugenol has a direct effect on ion channels expressed by peripheral sensory nerve fibers. Voltage-gated sodium channels are critical elements of action potential initiation and propagation in excitable cells, including sensory neurons, because they are responsible for the initial depolarization of the membrane (Hodgkin and Huxley, 1952). Therefore, it is possible that voltage-gated sodium channels are a molecular target for eugenol to elicit these effects.

Eugenol and capsaicin share the vanilloid moiety in their chemical structures (Sterner and Szallasi, 1999; Szallasi and Blumberg, 1999). Like capsaicin (Petersen *et al.*, 1989; Bleakman *et al.*, 1990), eugenol has inhibitory effects on voltage-gated calcium channel currents (I_{Ca}), and these effects might contribute to the analgesic effect of eugenol (Lee *et al.*, 2005). Interestingly, capsaicin requires transient receptor potential vanilloid 1 (TRPV1) for its inhibitory effect on voltage-gated calcium currents (Wu *et al.*, 2005), but eugenol does not (Lee *et al.*, 2005). Capsaicin also inhibits voltage-gated sodium channel currents (I_{Na}) only in capsaicin-sensitive trigeminal ganglion neurons (Liu *et al.*, 2001). Likewise, it is possible that eugenol might regulate I_{Na}. In the present study, we investigated the effect of eugenol on I_{Na} and its mode of action in rat dental primary afferent neurons, using a whole-cell patch-clamp technique.

MATERIALS & METHODS

All procedures for animal use were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the College of Dentistry, Seoul National University.

Preparation of Dental Primary Afferent Neurons

Dental primary afferent neurons were identified by retrograde labeling with a fluorescent dye (DiI: D-282, Molecular Probes, Eugene, OR, USA) as previously described (Lee *et al.*, 2005). Briefly, DiI was placed in the upper molar teeth of adult Sprague-Dawley rats (Samtako BioKorea, Inc., Osan-City, Korea) (200-250 g) under anesthesia. After 3 wks, trigeminal ganglia were digested with 0.25% trypsin at 37°C for 30 min, then cells were mechanically dissociated with a sterile Pasteur pipette and subsequently plated onto glass coverslips, previously coated by a solution of 0.1 mg/mL poly-L-ornithine. The trigeminal ganglion neurons were maintained in a humidified atmosphere of 95% O_2/5% CO_2 at 37°C and used for whole-cell recordings within 6 and 8 hrs.

Electrophysiological Recordings

Whole-cell current- and voltage-clamp recordings, respectively, were performed for the measurement of action potentials and I_{Na} with an Axopatch-1C amplifier (Axon Instruments, Union City, CA, USA). The patch pipettes were pulled from borosilicate capillaries (Chase Scientific Glass, Inc., Rockwood, TN, USA). When the pipettes were filled with the solution, their resistance was 2 ~ 4 MΩ. The pipette solution for current-clamp experiments was composed of (mM): K-gluconate 145, $MgCl_2$ 2, $CaCl_2$ 1, EGTA 10, HEPES 5, and K_2ATP 5, adjusted to pH 7.2 ~ 7.3 with KOH. Extracellular solution for current-clamp experiments contained (mM): NaCl 140, KCl 5, $MgCl_2$ 1, $CaCl_2$ 2, HEPES 10, and glucose 10, adjusted to pH 7.4 with NaOH. In current-clamp experiments, the resting potential was adjusted to -65 mV at the beginning of the experiment. Action potentials were evoked with 5-ms depolarizing current pulses with increasing amplitude (0.2 ~ 1 nA in 50.0-pA steps). Action potential duration was measured at 50% repolarization (APD_{50}). The pipette solution for I_{Na} was composed of (mM): CsCl 100, sodium L-glutamic acid 5, TEACl 30, $CaCl_2$ 0.1, $MgCl_2$ 2, EGTA 11, and HEPES 10, adjusted to pH 7.4 with CsOH. Extracellular solution for I_{Na} contained (mM): NaCl 90, choline chloride 30, TEACl 20, $CaCl_2$ 0.1, $MgCl_2\cdot5$, $CoCl_2$ 5, HEPES 10, and glucose 10, adjusted to pH 7.4 with NaOH. In voltage-clamp experiments, the I_{Na} was evoked by a test pulse to +0 mV from the holding potential, -80 mV every 10 sec. I_{Na} was classified into tetrodotoxin-sensitive (TTX-s) and tetrodotoxin-resistant (TTX-r) I_{Na}. For TTX-r I_{Na}, 1 μM TTX (Sigma, St. Louis, MO, USA) was used to block TTX-s I_{Na}. We obtained TTX-s I_{Na} by subtracting TTX-r I_{Na} from the total I_{Na}. In both voltage- and current-clamp experiments, series resistance was compensated for (> 80%), and leak subtraction was performed. Data were lowpass-filtered at 2 kHz, and sampled at 10 kHz. The pClamp8 (Axon Instruments) software was used during experiments and analysis. All the experiments were performed at room temperature.

Drugs

Capsaicin and capsazepine stock solutions were made in ethanol and stored at -20°C. Eugenol was dissolved in dimethylsulfoxide (DMSO) to make stock solution and kept at -20°C. All drugs were purchased from Sigma. 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 with 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. The bath solution perfusion was continuous during the experiment, at a rate of 2 mL/min.

Statistical Analysis

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

RESULTS

Eugenol Inhibited Action Potential in Both Capsaicin-sensitive and Capsaicin-insensitive Dental Primary Afferent Neurons

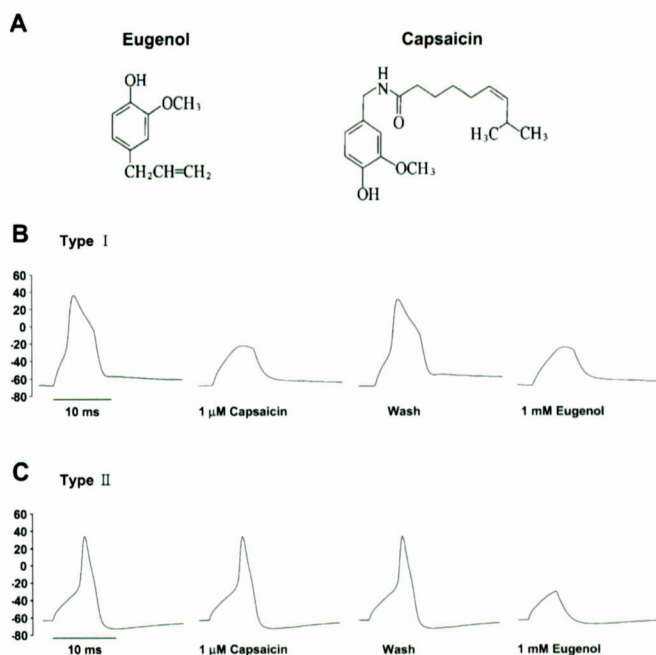Dental primary afferent neurons recorded in the study ranged

Figure 1. Effect of eugenol on action potentials in dental primary afferent neurons. At -65 mV, action potentials were evoked with 5-ms depolarizing current pulses with increasing amplitude (0.2 ~ 1 nA in 50.0-pA steps). Dental primary afferent neurons were classified into two types based on action potential shape: **(A)** The chemical structures of eugenol and capsaicin. Eugenol shares the vanilloid-moiety with capsaicin. **(B)** Type I neurons that had a prominent shoulder in the falling phase of the action potential were inhibited by capsaicin (n = 15). **(C)** Type II neurons that had no hump in the falling phase of the action potential were not affected by capsaicin (n = 15). Eugenol inhibited the generation of action potentials in both types of dental primary afferent neurons. B and C are representative responses of type I and type II neurons, respectively.

from 15 to 55 μm in diameter, and the resting membrane potential was -50 ± 10 mV (n = 72). Analogous to characteristics previously described in DRG neurons (Cardenas *et al.*, 1995), we distinguished two types of dental primary afferent neurons on the basis of their action potential duration, action potential shape, and their capsaicin sensitivity (Fig. 1). Type I neurons, mostly small (< 25 μm) and medium-sized neurons (25 ~ 35 μm), were characterized by long action potential duration (APD_{50} = 5.7 ± 0.5 ms), with a prominent shoulder in the falling phase (Fig. 1B). Type II neurons, mostly found in medium and large neurons (> 35 μm), had significantly shorter action potential durations (APD_{50} = 4.5 ± 0.6 ms) than did type I neurons (Fig. 1C). These type II neurons exhibited a small shoulder in the falling phase. A 1-μM concentration of capsaicin inhibited the generation of action potentials in type I neurons (n = 15), but not in type II neurons (n = 15). In contrast, 1 mM eugenol inhibited the generation of action potentials in both capsaicin-sensitive type I (n = 15) and capsaicin-insensitive type II dental primary afferent neurons (n = 15).

Action Potential Blockade Produced by Eugenol is Due to its I_{Na} Inhibition

Because voltage-gated sodium channels are responsible for the initial depolarization phase involved in the generation of action
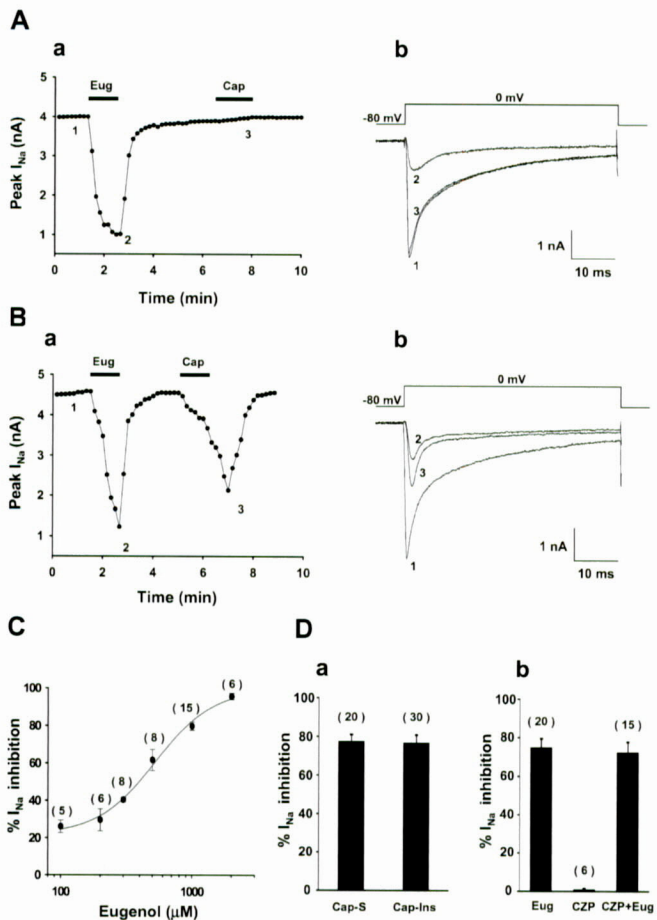
Figure 2. Effect of eugenol on voltage-gated sodium channel currents (I_{Na}) in dental primary afferent neurons. **(Aa)** Time-course of the effects of eugenol (1 mM) and capsaicin (1 μM) on I_{Na} in a capsaicin-insensitive neuron. Eugenol (1 mM) inhibited I_{Na} in capsaicin-insensitive neurons (n = 20). **(Ab)** Superimposed I_{Na} evoked by a test pulse at the points indicated in Aa. **(Ba)** The I_{Na} inhibition by eugenol was also observed in capsaicin-sensitive neurons (n = 30). **(Bb)** Superimposed I_{Na} evoked by a test pulse at the points indicated in Ba. A and B are representative traces illustrating the effect of eugenol on I_{Na} from capsaicin-insensitive and capsaicin-sensitive neurons, respectively. **(C)** Dose-response relationship of eugenol-induced I_{Na} inhibition. The inhibition of the peak I_{Na} by eugenol was dose-dependent with IC_{50} of 600 μM. **(Da)** The summary of I_{Na} inhibition in dental primary afferent neurons. Eugenol (1 mM)-induced I_{Na} inhibition in capsaicin-sensitive neurons (Cap-S) was similar to that obtained in capsaicin-insensitive neurons (Cap-Ins). **(Db)** The effect of capsazepine (10 μM), a competitive TRPV1 antagonist, on eugenol-induced I_{Na} inhibition. The I_{Na} inhibition by combined application of eugenol and capsazepine was not significantly different from that of eugenol (mean ± SEM, p > 0.05), indicating that eugenol-induced I_{Na} inhibition was TRPV1-independent. The numbers in parentheses represent the number of cells studied.

potential (Lai *et al.*, 2004), we explored the effect of eugenol on I_{Na}. A 1-mM concentration of eugenol inhibited I_{Na} in both capsaicin-sensitive neurons and capsaicin-insensitive neurons (Figs. 2A, 2B). The magnitude of I_{Na} inhibition by 1 mM eugenol was similar in capsaicin-insensitive neurons (77 ± 4%, n = 30) (Fig. 2Ab) and capsaicin-sensitive neurons (78 ± 3%, n = 20) (Figs. 2Bb, 2Da). I_{Na} inhibition by eugenol was dose-dependent (Fig. 2C, IC_{50} = 600 ± 75 μM). To test whether TRPV1 activation mediates the inhibitory effect of eugenol on
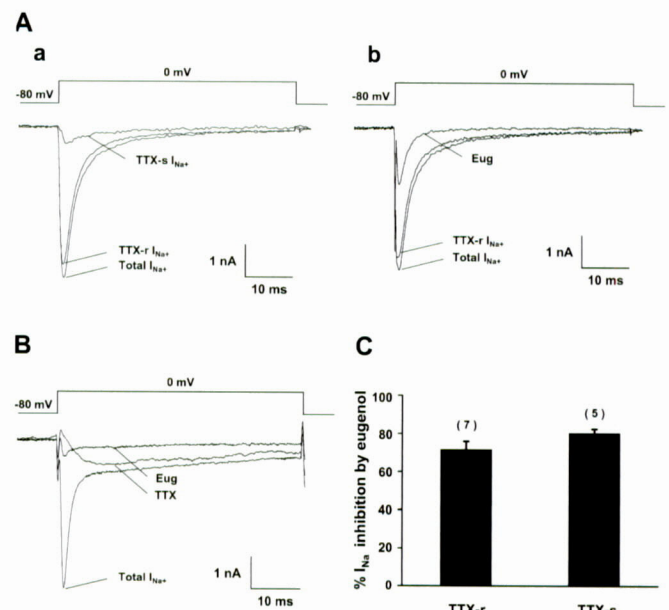

Figure 3. Effect of eugenol on two types of I_{Na} in dental primary afferent neurons. Both TTX-sensitive (TTX-s) and TTX-resistant (TTX-r) I_{Na} were present in dental primary afferent neurons. **(Aa)** TTX-r I_{Na} was predominant in small neurons (< 25 μm). TTX-s I_{Na} is the difference current of total I_{Na} and TTX-r I_{Na}. **(Ab)** TTX-r I_{Na} was blocked by 1 mM eugenol (n = 7). **(B)** TTX-s I_{Na}, which was predominant in large neurons (> 35 μm), was also blocked by 1 mM eugenol (n = 5). A and B are representative traces illustrating the effect of eugenol from TTX-r and TTX-s neurons, respectively. **(C)** The summary of TTX-r I_{Na} and TTX-s I_{Na} inhibition in dental primary afferent neurons. The extent of inhibition by eugenol on TTX-r I_{Na} was similar to that on TTX-s I_{Na} (mean ± SEM, p > 0.01). The numbers in parentheses represent the number of cells studied.

I_{Na}, we examined the effect of eugenol (1 mM) in the presence of capsazepine (10 μM), a competitive TRPV1 antagonist (n = 6). When we applied 10 μM capsazepine for 5 min, it did not produce any effect on the voltage-gated sodium channels, which was consistent with the previous report (Liu *et al.*, 2001). The I_{Na} inhibition by the combined application of eugenol and capsazepine (73 ± 5%, n = 15) was not significantly different from that of eugenol (75 ± 4%, n = 20) (Fig. 2Db).

Both TTX-s I_{Na} and TTX-r I_{Na} are Blocked by Eugenol

Two types of sodium channels, TTX-s and TTX-r, are expressed in sensory neurons (Wu and Pan, 2004). Consistent with this report, TTX-s I_{Na} and TTX-r I_{Na} were differentially distributed in the dental primary afferent neurons tested. Most of the I_{Na} was TTX-r I_{Na} in small neurons (< 25 μm), whereas TTX-s I_{Na} was predominant in large neurons (> 35 μm). TTX-s I_{Na} is the difference current of total I_{Na} and TTX-r I_{Na} (Fig. 3Aa). Eugenol significantly inhibited both TTX-r I_{Na} (Fig. 3Ab) and TTX-s I_{Na} (Fig. 3B). The magnitude of TTX-r I_{Na} inhibition by 1 mM eugenol (72 ± 4%, n = 7) (Fig. 3Ab) was similar to that of TTX-s I_{Na} (80 ± 3%, n = 5) (Figs. 3B, 3C).

DISCUSSION

Recently, we demonstrated that eugenol inhibits voltage-gated calcium currents in dental primary afferent neurons in

a TRPV1-independent manner (Lee *et al.,* 2005). It was thought that the inhibition of voltage-gated calcium channels might contribute to the analgesic effect of eugenol. In the present study, we further investigated whether eugenol would inhibit I_{Na} and its mode of action. We found that eugenol elicited an inhibitory effect on I_{Na}, thereby blocking the generation of action potential in dental primary afferent neurons, and this effect was observed in both capsaicin-sensitive and -insensitive neurons.

We found two types of action potentials in dental primary afferent neurons, as in other sensory neurons (Mirnics and Koerber, 1997; Lawson, 2002). Capsaicin-sensitive type I neurons, which have a relatively wide action potential with a shoulder on the falling phase, are likely to be nociceptive neurons. Capsaicin-insensitive type II neurons, exhibiting a narrow action potential but having no shoulder on the falling phase, could be non-nociceptive neurons. We observed that eugenol inhibited action potentials in both nociceptive and non-nociceptive neurons, regardless of their sensitivity to capsaicin. The extent of I_{Na} by eugenol in capsaicin-sensitive neurons was comparable with that in capsaicin-insensitive neurons, and capsazepine failed to block the eugenol-induced I_{Na} inhibition in dental primary afferent neurons. These findings suggest that TRPV1 activation might not be involved in the inhibitory effect of eugenol on I_{Na}. In contrast, capsaicin inhibits I_{Na} only in capsaicin-sensitive trigeminal ganglion neurons, and capsazepine, a competitive TRPV1 antagonist, inhibits capsaicin-induced blockade of I_{Na} in rat trigeminal ganglion neurons (Liu *et al.,* 2001). These observations are reminiscent of the effects of eugenol and capsaicin on I_{Ca}.

There are two general classes of sodium currents in sensory neurons: One is blocked by TTX (TTX-sensitive or TTX-s I_{Na}), and the other is insensitive to TTX (TTX-resistant or TTX-r I_{Na}). We found that eugenol inhibited both TTX-s I_{Na} and TTX-r I_{Na} in dental primary afferent neurons. Because TTX application to distal axons completely blocks conduction of action potentials (Ritter and Mendell, 1992; Brock *et al.,* 1998), it is clear that TTX-s I_{Na} mediate action potential conduction along both myelinated and unmyelinated axons. TTX-r I_{Na} were also found to mediate action potential initiation in polymodal nociceptive afferents (Brock *et al.,* 2001). In addition, TTX-r I_{Na} may contribute to the release of transmitter from the central terminals of nociceptive afferents (Gu and MacDermott, 1997). Thus, the inhibitory effect of eugenol on both TTX-s and TTX-r I_{Na} may contribute to its analgesic effect. We have recently reported that eugenol inhibited voltage-gated calcium currents (I_{Ca}) in dental primary afferent neurons (Lee *et al.,* 2005). The ranges of eugenol concentrations that produced inhibitory effects on I_{Na} (10^{-4} to ~ 10^{-3} M) were lower than those on I_{Ca} (~ 5 x 10^{-4} to ~ 10^{-3} M), indicating that voltage-gated sodium channels are more sensitive to eugenol than are voltage-gated calcium channels. Therefore, inhibition of action potential generation and propagation is likely to be a major molecular mechanism for the analgesic effect of eugenol.

It is interesting to note that, although eugenol and capsaicin share a vanillyl-like moiety in their chemical structure (Sterner and Szallasi, 1999; Szallasi and Blumberg, 1999), the mechanisms underlying inhibitory effects between eugenol and capsaicin on voltage-gated sodium channels and voltage-gated

calcium channels are different: One is TRPV1-independent, the other is TRPV1-dependent. These findings suggest that the vanillyl-moiety, which is known to be important for capsaicin-like action of vanilloid compound (Sterner and Szallasi, 1999; Szallasi and Blumberg, 1999), might not be the critical factor for determining mechanisms of voltage-gated channel regulation by vanilloid compounds, such as capsaicin and eugenol.

In summary, we demonstrate that eugenol produces an inhibitory effect on I_{Na} in dental primary afferent neurons, and that TRPV1 activation is not a prerequisite for the inhibitory effect of eugenol on I_{Na}. The inhibition of I_{Na}, in addition to I_{Ca}, is likely to be one of the molecular mechanisms underlying the analgesic effect of eugenol.

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