EFFECT OF EUGENOL AND CAPSAICIN ON THE VOLTAGE-DEPENDENT ION CHANNELS OF TRIGEMINAL AFFERENTS.

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ABSTRACT

삼차신경 일차구심 뉴런의 전압의존성 이온통로에 대한 capsaicin과 eugenol의 작용

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삼차신경절의 뉴런이 구강악안면영역에서의 촉각, 압각, 온도각 및 통각 등 다양한 감각을 중추신경계로 전달하는 역할을 하는 것은 주지의 사실이다. 이러한 신경전달에 있어서 이온통로는 감각정보를 전달하는데 핵심적인 역할을 수행하며 특히 소디움 통로는 활동전위의 발생에 중요하다. 소디움 통로는 tetrodotoxin-sensitive(TTX-s) 및 tetrodotoxin-resistant(TTX-r) 통로로 나누어지는 데 이 중 TTX-r 통로에 발생되는 tetrodotoxin-resistant sodium current(TTX-r I_{Na})는 capsaicin에 민감한 일차구심신경세포에서 유해자극에 의해 통각신호를 발생시키고 전달하는데 중요하다. 또한 칼슘 통로는 시냅스 전도에 있어서 필수적인 역할을 수행하고 있다. 한편 치과영역에서 치수의 진정 목적으로 eugenol이 흔히 사용되고 있다. 그러나 eugenol의 그 작용 기전에 대해서 현재까지 이온 통로에 대한 상세한 결과가 없는 실정이며 최근의 보고에 의하면 eugenol이 capsaicin 수용기를 통하여 감각신경에 대한 억제작용을 나타낸다고 한다. 따라서 본 실험은 eugenol과 capsaicin이 흰쥐의 삼차신경절의 TTX-r I_{Na}와 칼슘통로에 어떠한 영향을 미치는지를 알아보고 eugenol이 capsaicin 수용기를 통하여 작용하는지를 검증하고자 시행되었다. 삼차신경절 뉴런은 100~150g의 흰쥐의 삼차신경절로부터 외과적으로 절제하여 통법의 화학적 및 기계적 처리를 통해 단일세포로 분리하였고 이를 whole-cell patch clamp 방법을 이용하여 시행한 바 다음과 같은 결론을 얻었다.

1. 1mM의 dugenol은 흰쥐 삼차신경절 뉴런의 TTX-r I_{Na}와 HVA I_{Ca}를 억제하였다.
2. 1㎛의 capsaicin은 흰쥐 삼차신경절 뉴런의 TTX-r I_{Na}와 HVA I_{Ca}를 억제하였다.
3. Capsazepine은 capsaicin의 HVA I_{Ca}에 대한 억제작용을 차단하였다.
4. Capsazepine은 capsaicin의 HVA I_{Ca}에 대한 억제작용을 차단하지 못하였다.

결론적으로 eugenol과 capsaicin은 tetrodotoxin-resistant sodium current(TTX-r I_{Na})와 high voltage-activated calcium current(HVA I_{Ca})를 모두 억제하는 것으로 나타났으며, 이러한 작용이 통각의 발생과 시냅스 전달과정을 차단하여 치수 진정 목적으로 많이 사용하는 eugenol의 작용기전으로 판단된다. 한편 capsaicin의 길항제인 capsazepine을 전처치하였을 때에도 eugenol의 HVA I_{Ca}에 대한 억제효과는 변화가 없었다. 이와같은 결과로 보아 HVA I_{Ca}에 관한 한 eugenol은 capsaicin 수용기를 통하여 나타나지 않는 것으로 사료된다.

주요단어 : 삼차신경절, eugenol, capsaicin, TTX-r I_{Na}, HVA I_{Ca}, whole-cell patch clamp.

Ⅰ. INTRODUCTION

Eugenol, 4-allyl-2-methoxyphenol, has general toxic effects[1], cytotoxic effects[2], and local anesthetic and general anesthetic effects[3]. It causes peripheral vasodilation[4], enhances healing of inflamed tooth pulps[5], has allergenic potential[6] and causes inhibition of respiration in liver mitochondria[7].

Eugenol is used extensively in dentistry and is a common component of medicaments placed in the pulp cavities of teeth. Instances have been reported of such medicaments based on zinc oxide-eugenol being associated with paraesthesia of mandibular nerve[8] presumably by being pressed out through the root canals. In-vitro tests have demonstrated neurotoxic properties of such medicaments[9]. Eugenol is often used because of its anodyne effect of pulp-dentin organ, but little is known about the mechanism of this effect. The analgesic action of eugenol had long been attributed to the action as a non-specific counter-irritant. More recently, it has been reported that the agent inhibits sensory nerve activity[10,11], or has a potent inhibitory action on PGI2 production[12].

On the other hand, it has also been reported that eugenol possesses a capsaicin-like action on peripheral endings of primary afferents of the rat urinary bladder[13]. Capsaicin (8-methyl-N- vanillyl-6-nonemamide) is the pungent component of peppers and similar plants which has a variety of effects on sensory neurones[14]. Capsaicin has been known to cause the release and depletion of neuropeptides[15,16], cell excitation and fiber discharge[17], and neuronal cell death at high concentrations[18]. In rat dorsal root ganglion cells (DRG), capsaicin has been shown to cause cell depolarization[19] and increased cell excitability[20] as well as an increase in the accumulation of ^{45}Ca[21]. When using relatively low concentrations of capsaicin (<10 μM), these responses seem to be restricted to the C-type sensory cells responsible for the transmission of nociceptive information[22]. In this respect, capsaicin is an important tool for investigating the mechanism by which noxious stimuli are detected and sensory information is transmitted. The inorganic dye, ruthenium red (RR), which is an inhibitor of mitochondrial and Ca^{2+} transport [23,24], has also recently been shown to antagonize capsaicin-dependent increases in ^{45}Ca uptake[21], transmitter release[25], and primary afferent neuron excitation[26].

As for the antinociceptive action of capsaicin, it has been suggested that capsaicin-induced antinociception may also be mediated through a capsaicin receptor mechanism based on the fact that the inhibitory effect of capsaicin on nociceptive thresholds is significantly antagonized by capsazepine[27-29]. And a recent study reported that intrathecally injected eugenol exhibited the inhibitory action on nociceptive response, and capsazepine abolished the antinociceptive effects of eugenol[30]. This observation suggested that eugenol may act to reduce nociceptive thresholds via the capsaicin receptor located on sensory terminals in the spinal cord.

In the present study, we used eugenol, capsaicin, and capsazepine to investigate (1) their action on the tetrodotoxin-resistant sodium current (TTX-r I_{Na}) and high voltage-activated calcium current (HVA I_{Ca}), (2) the possible involvement of capsaicin receptor in the antinociceptive effects of eugenol in the rat trigeminal root ganglion (TRG) neurons.

Ⅱ. MATERIALS & METHODS

Preparation of rat TRG neurons

Rat TRG neurons were prepared by the method described by Liu and Simon (1996) as follows: after the decapitation of the ketamine- anesthesized (100~125mg/kg, I. M.) adult Sprague-Dawley rat (100~150g), a pair of trigeminal ganglia were dissected and washed several times in cold (4℃) modified Hanks' balanced salt solution (HBSS) containing 130NaCl, 5KCl, 0.3KH_2PO_4, 4$NaHCO_3$, 0.3 Na_2HPO_4, 5.68D-glucose and 10 *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid (HEPES) (in mM, pH 7.3). They were incubated for 40 minutes at 37℃ in HBSS containing 1mg/ml of type XI collagenase, triturated with flamed Pasteur pipettes, and finally incubated at 37℃ for 6min with 1mg/ml of type IV DNase I. Then they were retriturated and washed/centrifuged three times in Dulbecco's modified Eagle medium (DMEM), and maintained in room temperature. Cells were used in electrophysiological recording within 6 hours.

Recording solutions & Drugs

The composition of internal solution used for the isolation of I_{Na} was 110CsF, 10NaCl, 0.1CaCl2, 10 HEPES and 11 ethylene glycol-bis(β-aminoethyl ether)-N, N, N′, N′-tetraacetic acid (EGTA) (in mM, pH7.3 adjusted with CsOH). The external solution used for I_{Na} contained 60 choline-Cl, 60NaCl, 20 tetraethylammonium (TEA)-Cl, 5KCl, 5MgCl2, 2 CaCl2, 10HEPES and 20glucose (in mM, pH7.4 adjusted with NaOH). 0.1mM CdCl2 was added to this solution to suppress I_{Ca}. The internal solution used for I_{Ca} consisted of 100 Cs-methanesulfonate (Cs- eSO3), 0.1CaCl2, 20TEA-Cl, 10HEPES, 11 EGTA and 2Mg-ATP (in mM, pH7.3 adjusted with CsOH). The external solution used for ICa consisted of 140choline-Cl, 2MgCl2, 10CaCl2, 10HEPES and 20glucose (in mM, pH7.4 adjusted with CsOH). All the enzymes and chemicals above were purchased from Sigma, except DMEM and methanesulfonic acid from Gibco Laboratory and Fluka, respectively. TTX (Research Biochemical International) was dissolved first in ethyl alcohol and then diluted in the external solution before use. A stock solutions of capsaicin (Sigma), and capsazepine (Biomol Research Laboratory) were made by dissolving them in ethanol. And eugenol (Sigma) was directly dissolved into the external solution.

Electrophysiology

The isolated cells were plated onto a polyethyleneimine-coated glass coverslip in a recording chamber (100㎕ volume) for superfusion (3ml/min) with control and drug- containing solutions. Patch clamp pipettes were manufactured from soda lime glass using a two-stage vertical pipette puller (L/M-3P-A, List electronics), and fire-polished using a microforge (MF-83, Narishige). Due to large currents, low-resistance pipettes (around 0.7~1.5MΩ) were employed to reduce the voltage drop across the series resistance which was compensated 70~75% usually. Recording of the currents were performed when the resultant series resistance was less than 3MΩ after compensations. Whole-cell patch clamp recordings were performed with an Axopatch 200A patch clamp amplifier (Axon instrument). Stimulus application and data acquisition were controlled by an IBM-compatible personal computer in conjunction with a Labmaster (PP-50) DMA interface (Warner instrument). The data were lowpass filtered at 5kHz (-3 dB) with the amplifier's Bessel filter, and digitized every 20㎲. All data collection and analysis was carried out with pCLAMP 6.0 software (Axon instrument).

Neuron diameter was measured with an eyepiece micrometer under phase contrast illumination, and categorized into three groups: small (20~27㎛), medium (33~38㎛) and large (45~51㎛) cells. In the present study, we recorded voltage-dependent ion currents primarily in the small cells of 15~30㎛ in diameters without or with short processes since (1) they are known to be related to pain sensation[31], (2) the amplitude of current recorded from the medium or large cells was so great that a serious series resistance error and the saturation of amplifier could not be avoided under the ionic condition in this experiment (i. e. 60 Na^+ in external bathing solution), (3) the complete compensation of cell membrane capacitance was difficult frequently and the signs of poor space-clamp condition such as a slow deactivating current used to be detected in some medium and large cells or cells with processes.

All experiments were carried out at room temperature (22~25℃).

Ⅲ. RESULTS

Tetrodotoxin-resistant sodium current (TTX-r I_{Na})

I_{Na} was isolated from the other kinds of voltage-dependent currents in whole-cell patch clamp recordings by 1) suppressing IK with the use of the equimolar replacement of K^+ with Cs^+ in the internal solution and by the addition of 20mM TEA in the external solution, 2) suppressing ICa by 0.1mM Cd^{2+} in the external solution and 5mM Mg^{2+} in the internal and external solutions, and 3) decreasing the concentration of Na^+ in the external solution to 60mM by the isomolar substitution of choline to prevent the saturation of the amplifier by large I_{Na}. Inward current recorded under this ionic condition was reduced as expected by the partial replacement of the external Na^+ by choline (Fig. 1A). The observed potential for zero current flow was 46.7±0.5 mV

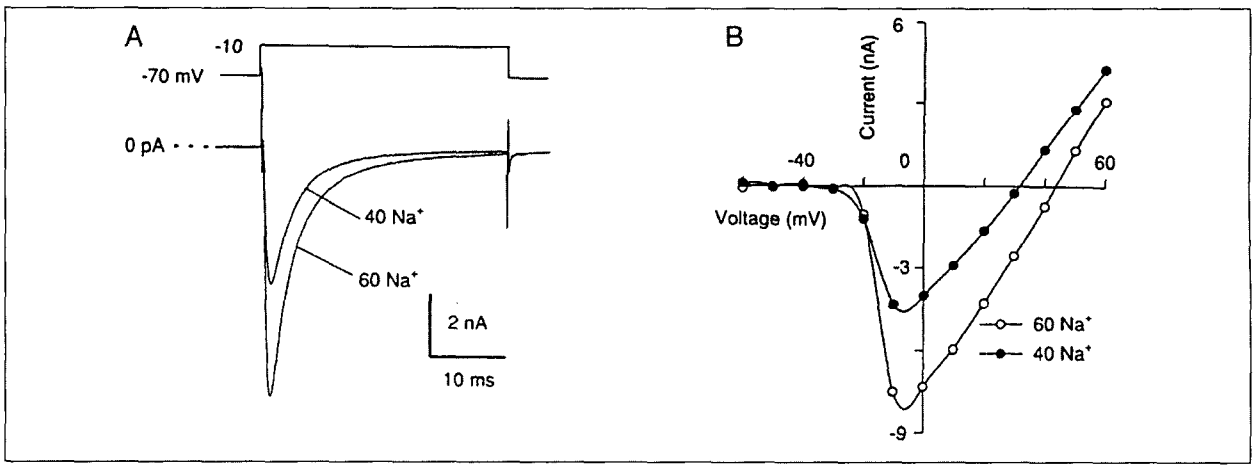

Fig. 1. Inward current component carried by Na^+

(n=30), as expected for sodium selective channel, and moved 11.3±0.3mV (n=3) in the hyperpolarizing direction when the external Na^+ concentration was reduced from 60 to 40 mM (Fig. 1B), which is identical to the predicted value by the Nernst equation for a sodium-selective channel. In addition, when the Nav in the bathing solution were totally removed, the current traces became much smaller and the direction of the current became outward (n=3, not shown). These observations indicate that the inward current may be voltage dependent I_{Na}.

Fig. 2A shows typical I_{Na} recorded from the acutely isolated rat TRG neuron under the ionic condition described above. I_{Na} was first observed at the potential of -40~-30mV and peaked at -20~+10mV (Fig. 2A). The current was appeared to be composed of two components of voltage-dependent I_{Na} distinguished by their sensitivity to TTX. In most cases, 0.1μM TTX failed to inhibit INa completely (Fig. 2A(b)). The component which was resistant to 0.1μM TTX was not also significantly inhibited by 20μM TTX (n=2, not shown). TTX-r I_{Na} was observed in the 〉90% of the small TRG neurons less than 30μm in diameter in the presence of 0.1μM TTX. However, external application of 0.1μM TTX suppressed most of the current in some preparations (Fig. 2B). This suggests that most of the small TRG neurons from adult rat may express TTX-r I_{Na} with or without TTX-s I_{Na}, which resulted in difficulties in direct observation of isolated TTX-s I_{Na} in small neurons.

The maximal current density of the TTX-r I_{Na} was quite various from cell to cell. The maximal TTX-r I_{Na} was elicited at a test potential of 0 or +10mV (Fig. 2Ad), and the current density of TTX-r I_{Na} elicited by voltage steps to +10mV from a holding potential (Vh) of 70mV was 268.5 20.6 pA/pF (n=20).

EFFECTS OF EUGENOL&CAPSAICIN ON TTX-r I_{Na}

In this study, the actions of eugenol and capsaicin on the TTX-r I_{Na} in the presence of 2mM Ca^{2+}, 5mM Mg^{2+} and 0.1mM Cd^{2+} in the external bathing solution were investigated. 1mM Eugenol and 1μM capsaicin were tested. For the evaluation of the effects of both eugenol and capsaicin, a constant 8 ms step pulse of 0mV from 70mV was stimulated before and after perfusion of the agents. Current traces were leak-subtracted and the current amplitude was measured at the peak of the current. Fig. 3 shows TTX-r I_{Na} evoked by a step pulse of 0mV after perfusion of 1 mM eugenol. Amplitude of TTX-r I_{Na} evoked by a step pulse of 0mV in control was about 3nA, and 1mM eugenol blocked TTX-r I_{Na} up to 50% of that of control. On the other hand 1μM capsaicin also blocked about 30% of TTX-r I_{Na} and its inhibitory effect was less than that of eugenol (Fig. 4). Also we evaluated the effect of eugenol and capsaicin on the voltage dependence of TTX-r I_{Na}. TTX-r I_{Na} was evoked by a ramp pulse from -70mV to +20mV. During the period of the ramp pulse 1mM Eugenol and 1μM capsaicin partially blocked the TTX-r I_{Na} (Fig. 5). And the voltage dependence of TTX-r I_{Na} was little changed after perfusion of eugenol and capsaicin (Fig. 6).

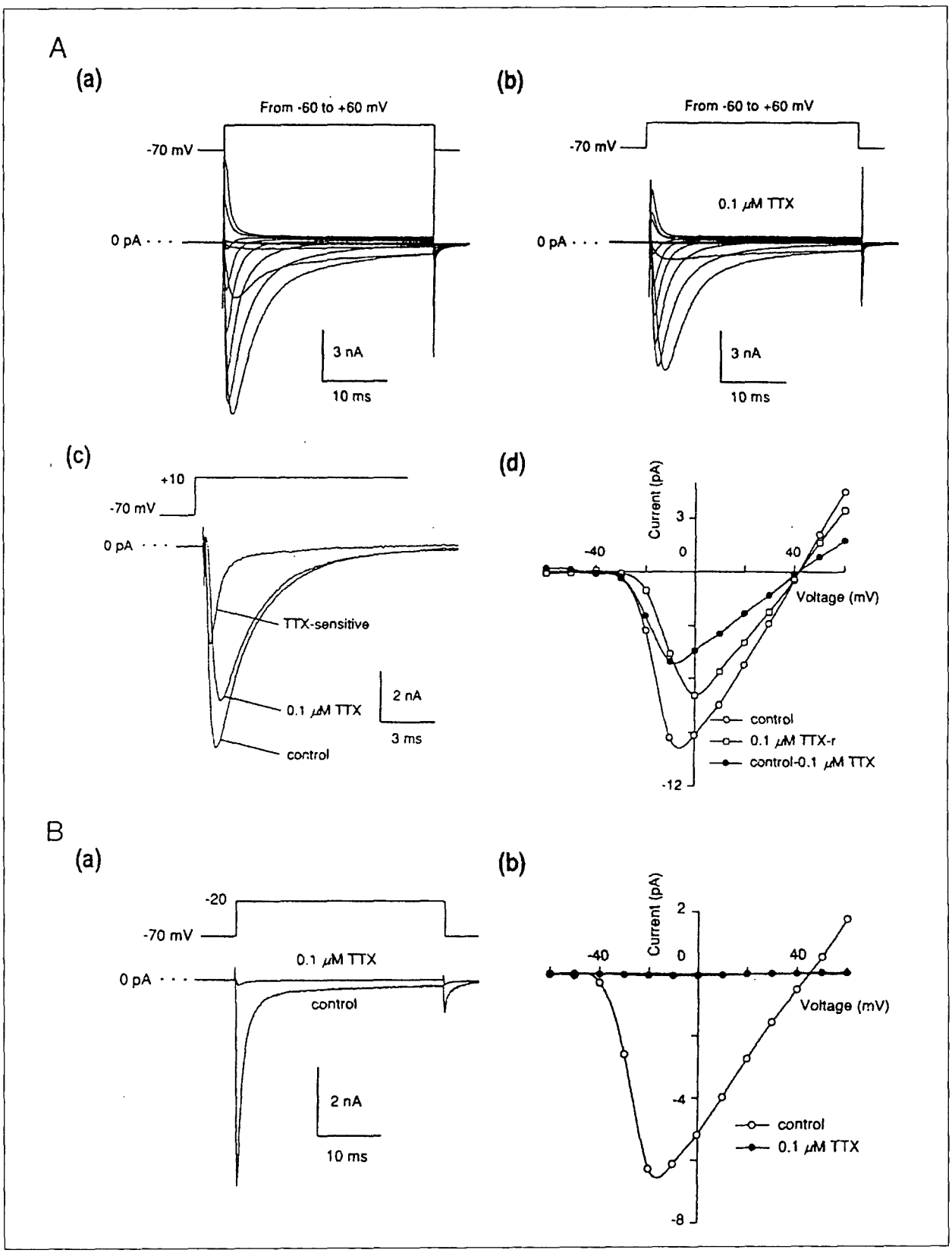

Fig. 2. Two types of I_{Na} ; TTX-s and TTX-r I_{Na}.

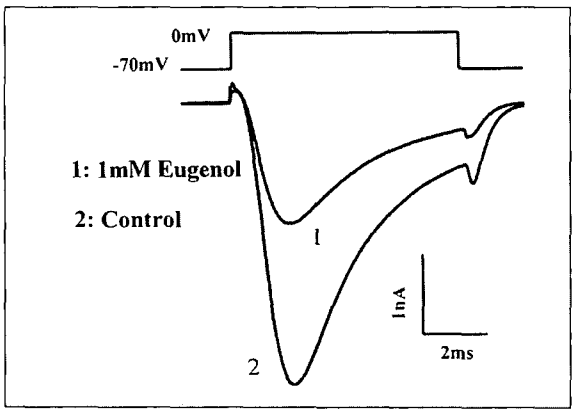

Fig. 3. Eugenol inhibited TTX-r I_{Na} of TRG afferent

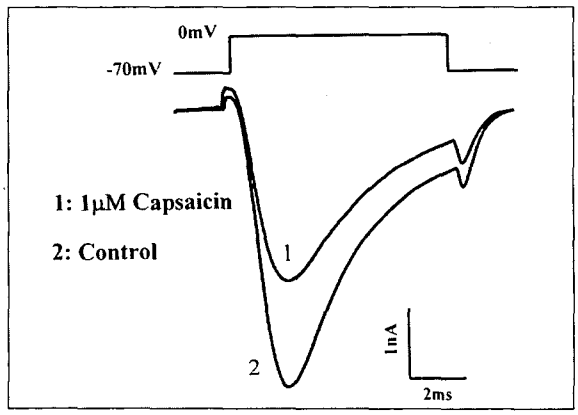

Fig. 4. Capsaicin inhibited TTX-r I_{Na} of TRG afferent

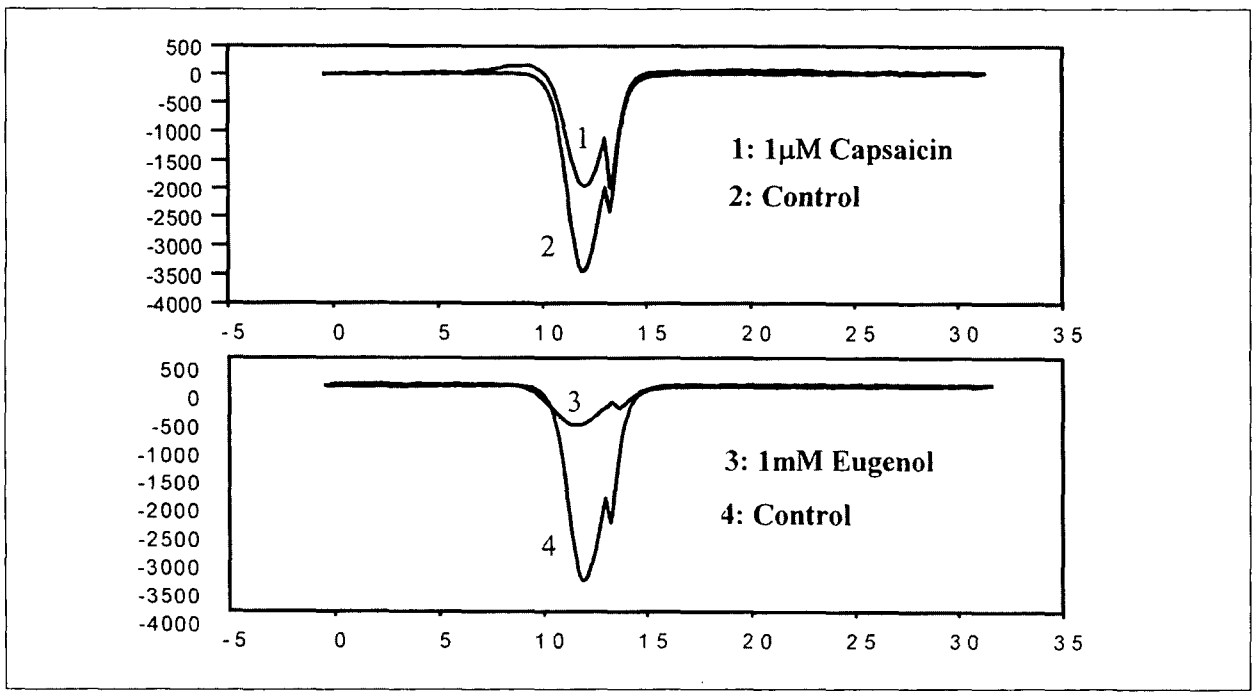

Fig. 5. Eugenol & capsaicin inhibited TTCX-r I_{Na} evoked by ramp pulse

HIGH VOLTAGE-ACTIVATED CALCIUM CURRENT (HVA I_{Ca})

I_{Ca} was recorded from rat TRG neurons under conditions which 1) suppressed IK by the replacement of internal K^+ with Cs^+, and 20mM TEA included in external solution, 2) suppressed I_{Na} with choline replacing external Na^+, and 3) enhanced I_{Ca} using 10mM Ca^{2+} as a charge carrier.

Fig. 7 shows whole-cell I_{Ca} obtained from a small diameter neuron evoked by depolarizing steps to various test potentials from a Vh of -80mV, which is thought to be comprised of multiple components of I_{Ca}. By applying depolarizing steps from a Vh at -80 mV, transiently inactivating I_{Ca} was first observed at about -60mV. The current reached its peak in 5~30 ms after the onset of the step pulse and decayed rapidly in a single-exponential time course with a time constant of 15~30ms. With further depolarization the peak amplitude increased smoothly and reached a plateau at about -30mV, resulting in a distinct shoulder in I-V relationship (Fig. 7C). At the

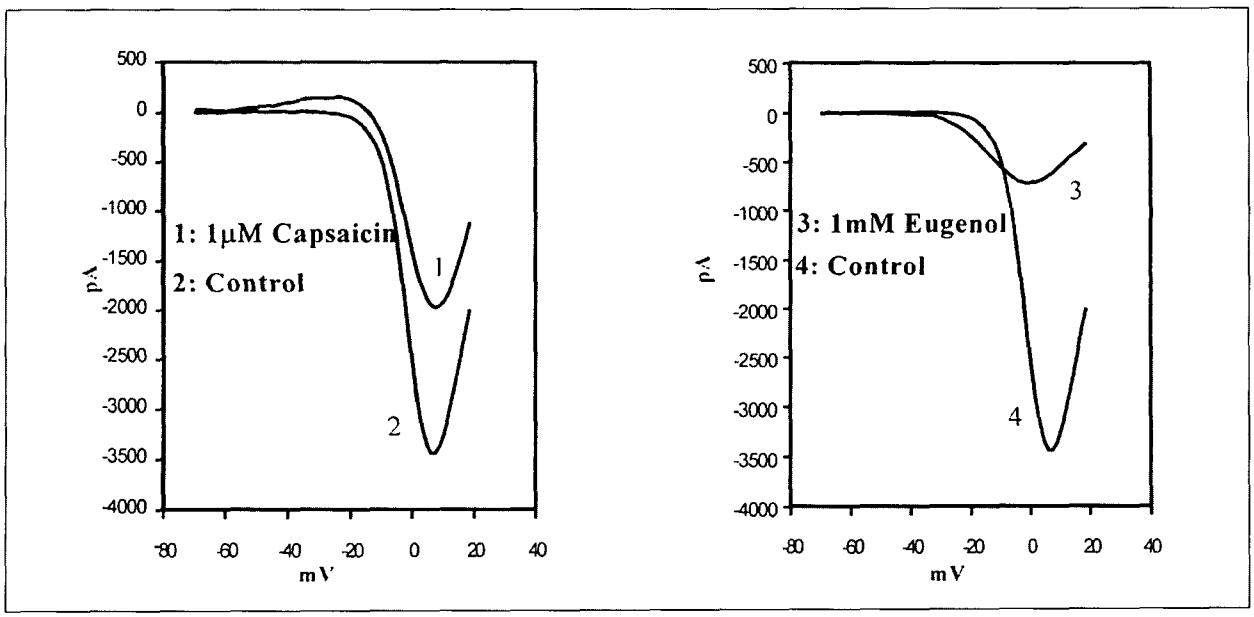

Fig. 6. I-V relationship of TTX-r I_{Na} with capsaicin & eugenol

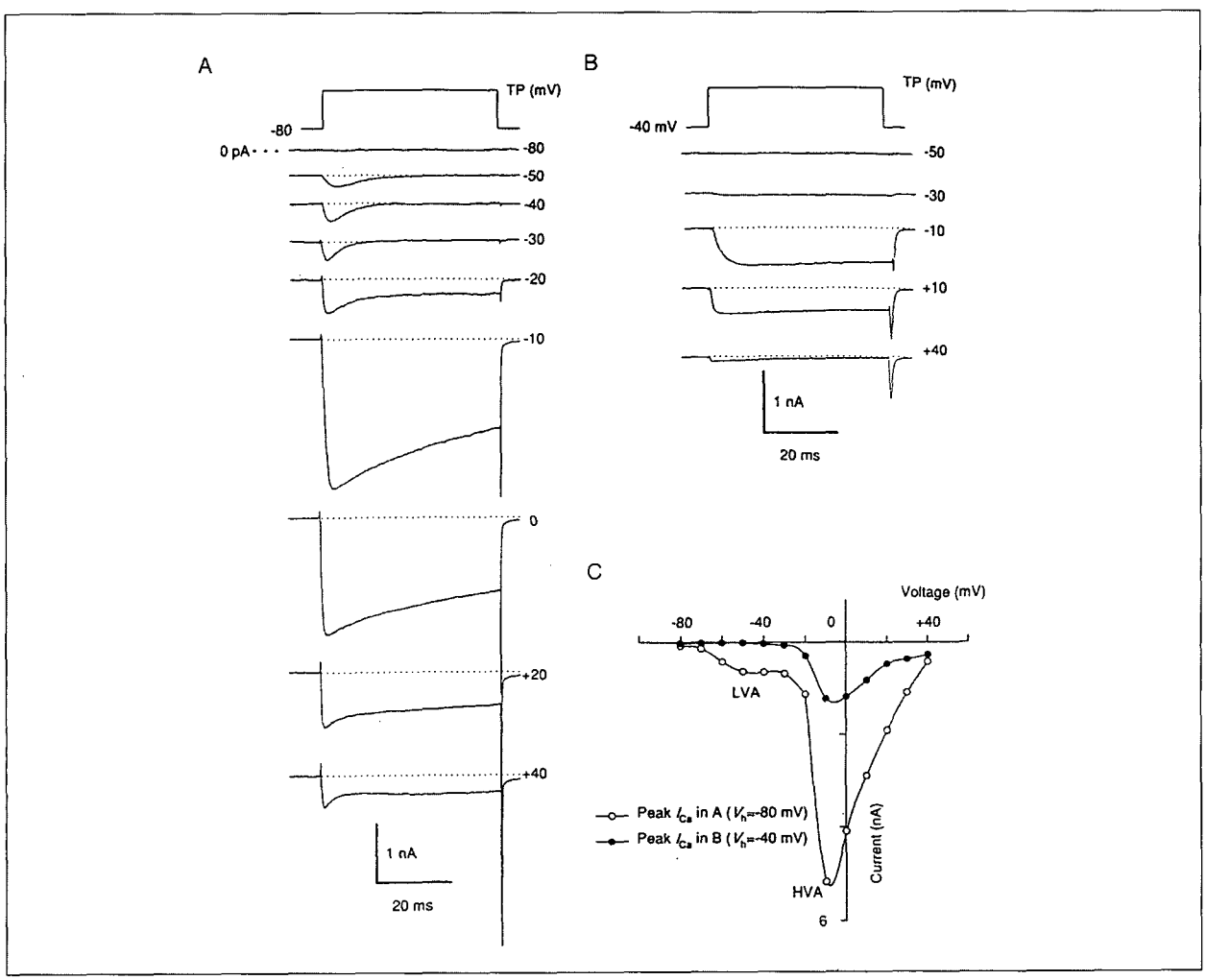

Fig. 7. Three components of I_{Ca} observed I a rat trigeminal root ganglion(TRG) neuron

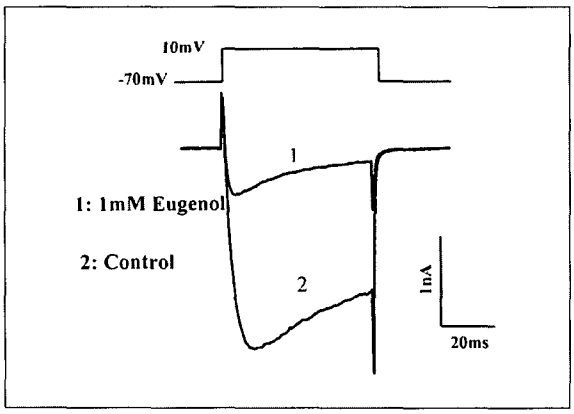

Fig. 8. Eugenol inhibited HVA I_{Ca} of TRG afferent

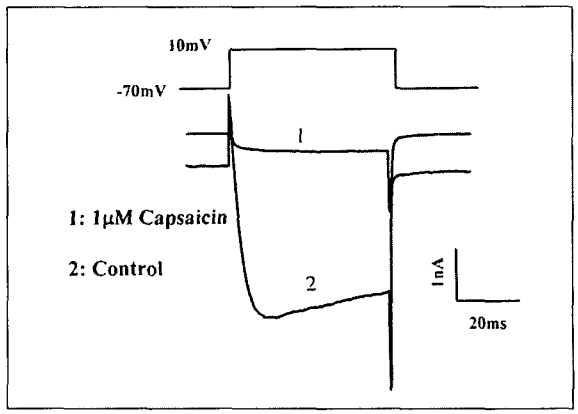

Fig. 9. Capsaicin inhibited HVA I_{Ca} of TRG afferent

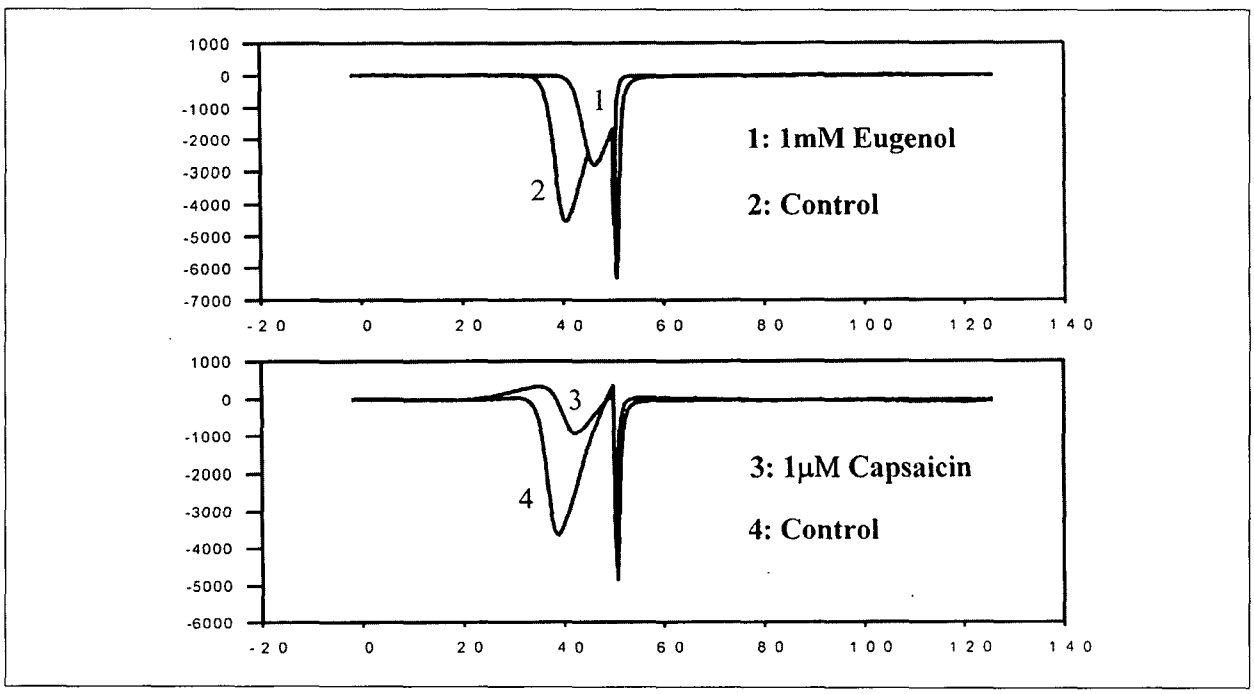

Fig. 10. Eugenol & capsaicin inhibited HVA I_{Ca} evoked by ramp pulse

potential about -30~-20mV, a step increase in current amplitude was observed and the decay of the current became progressively less pronounced. When the cell was held at -40mV, all of the decaying component of I_{Ca} could no longer be evoked over the whole range of test potentials, but sustained I_{Ca} was activated at the stronger depolarizations. The current showed little inactivation during 80ms step pulse (Fig. 7B) and was maximal at near -10mV (Fig. 7C). These observations suggest the presence of two current components with different activation range, i.e. low-voltage activated (LVA) and high-voltage activated (HVA) I_{Ca}, in small sized rat TRG neurons.

EFFECTS OF EUGENOL & CAPSAICIN ON HVA I_{Ca}

For the evaluation of the effects of both eugenol and capsaicin on HVA I_{Ca}, a constant 50ms step pulse of 10mV from -70mV was stimulated before and after perfusion of the agents. Current traces were not leak-subtracted and the current amplitude was measured at the peak of the current. Fig. 8 shows HVA I_{Ca} evoked by a step pulse of 10mV after perfusion of 1mM eugenol. Amplitude of HVA I_{Ca} evoked by a step

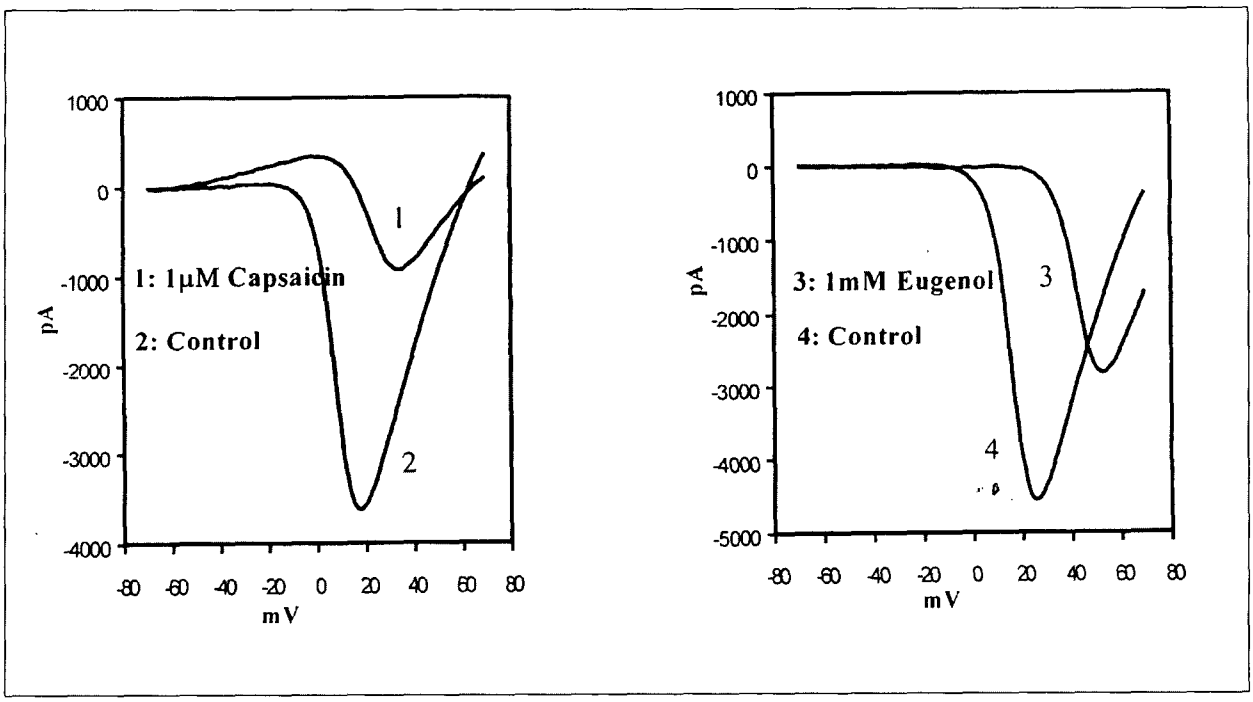

Fig. 11. I-V relationship of HVA I_{Ca} with capsaicin & eugenol

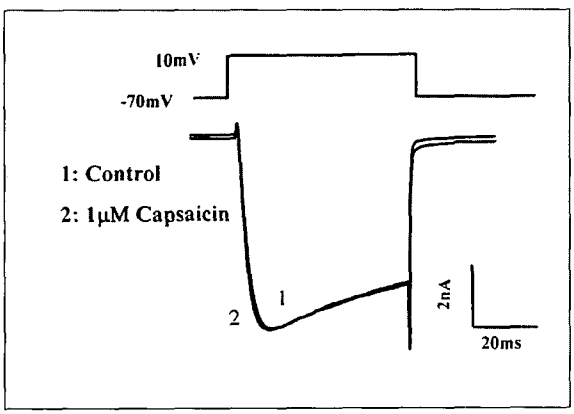

Fig. 12. Capsazepine blocked the effect of capsaicin on the HVA I_{Ca}

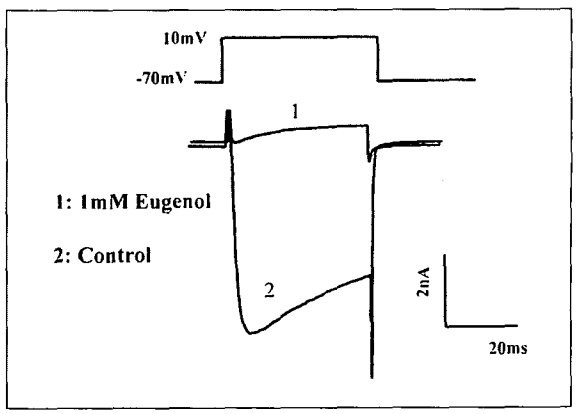

Fig. 13. Eugenol blocked HVA I_{Ca} with capsazepine

pulse of 10mV in control was about 2.2nA, and 1 mM eugenol blocked HVA I_{Ca} up to 75% of that of control. Also 1μM capsaicin blocked about 90% of HVA I_{Ca} (Fig. 9). And we evaluated the effect of eugenol and capsaicin on the voltage dependence of HVA I_{Ca}. HVA I_{Ca} was evoked by a ramp pulse from -70mV to +70mV. During the whole period of the ramp pulse 1mM eugenol and 1μM capsaicin markedly blocked the HVA I_{Ca} (Fig. 10). Fig. 11 shows the current-voltage (*I-V*) relationship of theHVA I_{Ca}. There was a significant shift in the (*I-V*) relationship of the HVA I_{Ca} after perfusion of eugenol and capsaicin. 1mM Eugenol and 1μM capsaicin shifted the (*I-V*) relationship of the HVA I_{Ca} to a depolarizing direction. This means that eugenol and capsaicin can alter the voltage dependence of HVA I_{Ca} of TRG neurons.

EFFECT OF CAPSAZEPINE ON HVA I_{Ca} BLOCKADE

Capsazepine, a selective and competitive antagonist of the actions of capsaicin, was used to investigate

the possible involvement of capsaicin receptor in the inhibitory effects of eugenol on the HVA I_{Ca}. For the evaluation of this relationship, TRG neurons were perfused with calcium external solution contained 50 μM capsazepine and stimulated by a constant 50ms step pulse of 10mV from -70mV before and after perfusion of eugenol and capsaicin. Current traces were not leak-subtracted and the current amplitude was measured at the peak of the current. Fig. 12 shows HVA I_{Ca} evoked by a step pulse of 10mV after perfusion of 1μM capsaicin. The current trace in control was identical to that evoked by capsaicin. This indicated that capsazepine blocked the effect of capsaicin on the HVA I_{Ca}. However eugenol blocked the HVA I_{Ca} evoked by a step pulse of 10mV in the presence of 50 μM capsazepine, or capsazepine did not affect the inhibitory effects of eugenol on the HVA I_{Ca} (Fig. 13). This means that eugenol may act on receptors that are not capsaicin receptors of TRG neurons in relation with the HVA I_{Ca}.

Ⅳ. DISCUSSION

The voltage-sensitive Na^+ channel, whose opening causes the upstroke of the nerve action potential, is blocked in practically all neurons by low concentrations of TTX. Na^+ channels in nociceptive neurons have long been known to be an exception as they are TTX-resistant, a property they share with Na^+ channels in cardiac muscle[32,33]. A TTX-resistant Na^+ channel has recently been isolated by difference cloning of mRNA from the dorsal ganglion (DRG)[34]. Patch clamp techniques have been used to investigate the range and properties of ionic channels underlying these electrical activity in dissociated mammalian DRG neurons, and two types of I_{Na} have been characterized : in general, TTX-s I_{Na} are found in large-diameter cells, and TTX-r I_{Na} are expressed in small-diameter cells[32,35-37,38-40]. Although the functional contribution of TTX-r I_{Na} to the electrophysiological activities of sensory neurons is not clear, it is suggested that the TTX-r I_{Na} may contribute to the prolongation of the interspike interval by extending the inactivated state of TTX-s I_{Na} during sustained depolarization of the membrane potential[41] or may be responsible for the slow adaptive properties and the generation of the long trains of action potentials because TTX-r I_{Na} shows the extremely slow inactivation process and the rapid repriming kinetics[32,42]. Recent studies suggested that TTX-r I_{Na} may participate in the sensitization to noxious stimuli in small capsaicin-sensitive primary afferent neurons, based on its modulation by pain-inducing agents[43]. We recorded I_{Na}, primarily, from the small sized TRG neurons ranging from 15 to 30μm and we observed TTX-r I_{Na} in every cell investigated in the presence of 0.1μM TTX.

Calcium channels help control many biological functions including excitability, transmitter release, contraction, metabolism and gene expression. Thus, there is great interest in the existence and properties of different types of Ca^{2+} channels[44,45]. The co-existence of multiple types of Ca^{2+}-selective channels within a given type of cell was first described on the basis of voltage-clamp studies utilizing microelectrode recordings[46]. In vertebrate neurons, multiple Ca^{2+} conductances were predicted initially on the basis of voltage recordings[47]. Subsequent voltage-clamp experiments demonstrated three or more populations of Ca^{2+} channels in many species and tissues.

Rat DRG neurons are known to have several types of calcium channels[48,49] which are similar to those reported in chick sensory neurons[50] : (1) a transient (T-type) or LVA ICa, (2) a dihydropyridine-sensitive, sustained (L-type) or HVA I_{Ca}, and (3) N-type I_{Ca} showing kinetic properties between the T- and L-type I_{Ca}, and a selective suppression by ω-CgTx. Recently, a fourth type, known as the P-type I_{Ca}, distinguished by its unique sensitivity to ω-agatoxin, also has been described in the rat DRG neurons[51]. We identified both LVA and HVA I_{Ca} from acutely isolated rat TRG neurons. LVA I_{Ca} was identified by the low threshold for the activation at about -60~-50 mV with maximal current amplitude at the voltage of -30mV, a fast rate of inactivation and a shoulder in the *I-V* curve. HVA I_{Ca} was recorded in every cell investigated, whereas LVA I_{Ca} did not appear to be recorded uniformly. Therefore we investigated the effect of eugenol and capsaicin on the HVA I_{Ca}.

Eugenol (as clove oil) has been widely used for many years as a topical, temporary treatment for the pain of pulpitis. In combination with zinc oxide, eugenol has found use as a temporary filling, lining, base, periodontal dressing, and pulp-capping materi-

al. It is still recommended for all but the last application. Sedative ad anodyne effects of the agent are recognized, but a concomitant irritant effect has been noted[52]. There is strong evidence that eugenol inhibits sensory nerve action[10,11,53], and that it inhibits the synthesis of prostaglandins, which are very probably involved in the inflammatory process[54]. Eugenol also inhibits cellular respiration, in a similar concentration range[55].

The depression of nerve excitability was obtained from the prevoius reports[10,11,53]. Brodin and Roed (1984)[56] reported that the inhibition of the nerve and muscle activity was reversible below 2.44mM, but irreversible at 2.44mM or higher concentrations of eugenol, and suggested that the analgesic effect of eugenol when applied to the pulp-dentine organ may be caused by reversible local anesthetic effects or irreversible inhibition of the excitability, depending on the concentration of eugenol surrounding the nerve fibers. Veaco et al. (1982)[57] found that the concentration of eugenol within the pulp cavity was about 10^{-5}M 8min to 8h after placement of a pellet with eugenol in a cavity in dentine. Thus, the anodyne effect on the pulp-dentine organ may be due to neurotoxic effect on the nerve fibers in dentine, and local anesthetic effects on the nerve fibers in the pulp.

The mechanisms for inhibition of nerve and muscle cell excitability by membrane stabilizing drugs are an inhibition of sodium current during the action potential[58,59]. However, more evidence that eugenol inhibits sodium ion current needs to be obtained by voltage clamp experiments. We used 1mM eugenol which is reversible concentration in the inhibition of nerve fibers, and it markedly inhibited the TTX-r I_{Na}. This finding strongly suggests that eugenol directly blocks the TTX-r sodium channels and then inhibits the generation of action potentials for the pain impulses in the maxillofacial region.

As mentioned above, voltage-activated calcium channels provide a link between electrical activity and intracellular chemical signaling. They are believed to play a crucial role in a number of cellular processes, especially neurotransmitter release. However, there is little effort to investigate the effect of eugenol on the voltage-gated calcium channels. In the present experiment eugenol significantly inhibited the HVA I_{Ca}. On the basis of the physiological function of the calcium channels, it is suggested that eugenol may inhibit the synaptic transmission as well as blocking the generaton of action potentials in the process of pain impulses.

Capsaicin is eaten daily by over a third of the world's population and produces its pungent taste sensation when it binds to receptors on a subset of nociceptors and warm thermoreceptors[20]. Upon prolonged application, it blocks nerve fiber conduction and depletes peptides from nerve terminals, which accounts for its clinical use as an analgesic and anti-inflammatory agent [60]. Capsaicin is thought to produce these effects by activating a single type of cation-selective ion channel present in a subset of nociceptive fibers[61].

Bleakman et al. (1990)[62] demonstrated that the rise in intracellular calcium concentration produced by exposing DRG cells to capsaicin results from Ca^{2+} entry from the extracellular solution rather than release of Ca^{2+} from intracellular store since it was abolished in a Ca^{2+}-free solution. For cells which were voltage-clamped at 80mV, the route of Ca^{2+} entry is likely to be via the established capsaicin-induced conductance which has previously been shown to be permeable to Ca^{2+}, Na^{+}, guanidinium and Rb^{+} ions[14,21]. Although Wood et al. (1988)[21] reported no reduction in capsaicin-dependent 45Ca fluxes into cultured DRG cells by dihydropyridine drugs, not all calcium channels in rat DRG neurons are dihydropyridine-sensitive[63]. Our finding showed that capsaicin blocked the HVA I_{Ca} considerably and this effect is similar to the other previous reports concerned with the effects of capsaicin on voltage-dependent calcium channels. In addition to this result, we investigate the effect of capsaicin on the TTX-r I_{Na} which is responsible for the generation of pain impulses in the nociceptors. Capsaicin inhibited the TTX-r I_{Na} expressed in the small neurons of TRG. Therefore we suggested that capsaicin blocks or inhibits the generation of action potentials, nerve conduction, and synaptic transmission of pain sensation.

The chemical structure of phenolic compound, such as eugenol is partly similar to that of capsaicin. Ohkubo and Shibata (1997)[30] tested the antinociceptive effect of eugenol and capsaicin to investigate the

receptors involved in their effect. In this experiment intrathecally injected eugenol and capsaicin produced the inhibitory action in a dose-dependent manner 24 h after treatment with the agents. In addition, dose-response curves for each of these compounds were shifted in a competitive manner by intrathecal capsazepine. Capsazepine, a selective and competitive antagonist of capsaicin, also abolished the antinociceptive effects of eugenol in the acetic acid writhing test. Ohkubo and Shibata (1997)[30] concluded that eugenol may act to reduce nociceptive thresholds and this effect may appear via the capsaicin receptor located on sensory terminals in the spinal cord.

In the present experiment, we tested whether eugenol acts on the capsaicin receptors or not. Our finding showed that eugenol still inhibited the HVA I_{Ca} in the presence of capsazepine. These data are therefore not consistent with the hypothesis of Ohkubo and Shibata (1997)[30] that eugenol may act on the capsaicin receptor located on sensory terminals in the spinal cord. And our result means that capsazepine did not affect the effect of eugenol on the high voltage-activated calcium channels and eugenol may act on the receptors that are not capsaicin receptors in its antinociceptive action

It is therefore concluded that eugenol and capsaicin inhibited the TTX-resistant voltage-gated sodium channels and high voltage- activated calcium channels in TRG neurons, and effect of eugenol on the high voltage-activated calcium channels may produce via other receptors different from capsaicin receptors.

V. CONCLUSION

Trigeminal root ganglia (TRG) neurons transmit various sensory information such as touch, pressure, pain and temperature from the oral & maxillofacial region to the central nervous system. Ionic channels in neuronal membranes have a fundamental role in the transmission of various sensory impulses. Sodium channels are important to generation action potentials and classified into TTX-r and TTX-s sodium channels. Calcium channels play a pivotal role in many biological functions including excitability, transmitter release, contraction, metabolism and gene expression. Especially, they have a fundamental importance in the synaptic transmission. Thus, there is great interest in the existence and properties of different types of Ca^{2+} channels. Eugenol is often used because of its anodyne effect of pulp-dentin organ, but little is known about the mechanism of this effect. As for the antinociceptive action of capsaicin, it has been suggested that capsaicin-induced antinociception may also be mediated through a capsaicin receptor. Therefore this experiment was performed to investigate the their action on the tetrodotoxin-resistant sodium current (TTX-r I_{Na}) and high voltage-activated calcium current (HVA I_{Ca}), and the possible involvement of capsaicin receptor in the antinociceptive effects of eugenol in the rat trigeminal root ganglion (TRG) neurons

It is concluded that eugenol and capsaicin inhibited the TTX-resistant voltage-gated sodium channels and high voltage-activated calcium channels in TRG neurons, and these results can explain the antinociceptive action of eugenol and capsaicin. On the other hand effect of eugenol on the high voltage-activated calcium channels may produce *via* other receptors different from capsaicin receptors.

REFERENCES

1. Sober HA, Hollander F, sober EK. Toxicity of eugenol : determination of LD50 on rats. *Soc.Exp. Biol. Med.73/74*: 148-151, 1950.
2. Kozam G, Mantell GM. The effect of eugenol on oral mucous membrane. *J. Dent. Res.* 57: 954-957, 1978.
3. Siemoneit KD, Zipf HF, Dittmann EC. Muntersuchungen zur endoanasthetischen und hypnotisch/narkotischen Wirkung von 2-Methoxy-4-allyl-phenoxy-essigsaure-N,N-diathylamid (G 29505) und verwandten Phenolderivaten. *Archs Int. Pharmacodyn. Ther.* 164:30- 46, 1966.
4. Stricht FD, Smith RM: Eugenol : some pharmacologic observations. *J. Dent. Res.* 50: 1531-1535, 1971.
5. Mjor I, Tronstad L. The healing of experimentally induced pulpitis. *Oral Surg.* 38: 115-121, 1974.
6. Koch G, Magnusson B, Nyquist G. Contact allergy to medicaments and material used in dentistry. II. Sensitivity to eugenol and colophony. *Odont. Revy* 22: 275-289, 1971.
7. Cotmore JM, Burke A, Lee NH, Shapiro IM. Respiratory inhibition of isolated rat liver mitochondria by eugenol. *Archs Oral Biol.* 24: 565-568, 1979.
8. Orstavik D, Brodin P, Aas E. Paraesthesia following endodontic treatment: survey of the literature and report of a case. *Int. Endodont. J.* 16: 167-172, 1983.
9. Brodin P, Roed A, Aars H, Orstavik D. Neurotoxic effects f root filling materials on rat phrenic nerve *in vitro*. *J. Dent. Res.* 61: 1020-1023, 1982.
10. Ozeki M. The effects of eugenol on the nerve and muscle in crayfish. *Comp. Biochem. Physiol* 50: 183-191, 1975.
11. Trowbridge H, Edwall L, Panopoulos P. Effect of zinc oxide-eugenol and calcium hydroxide on intradental nerve

activity. *J. Endo.* 8: 403-406, 1982.
12. Hirafuji M. Iinhibition of prostaglandin I2 biosynthesis in rat dental pulp by phenolic dental medicaments. *Jpn. J. Pharmacol.* 36: 544-546, 1984.
13. Patacchini R, Maggi CA, Meli A. Capsaicin-like activity of some natural pungent substances on peripheral endings of visceral primary afferents. *Naunyn-Schmiedelberg's Arch Pharmacol.* 342: 72-77, 1990.
14. Bevan S, James IF, Rang HP, Winter J, Wood JN. The mechanism of action of capsaicin-a sensory neurotoxin. In *Neurotoxins and their pharmacological implications.* Ed. Jenner P. New York: Raven Press. 1987.
15. Gamse R, Lackner D, Gamse G, Leeman SE. Effect of capsaicin pretreatment on capsaicin-evoked release of immunoreactive somatostatin and substance P from primary sensory neurons. *Naunyn-Schmiedelberg's Arch Pharmacol* 316: 38-41, 1981.
16. Fitzgerald M. Capsaicin and sensory neurones. *Pain* 15: 109-130, 1983.
17. Foster RW, Ramage AG. The action of some chemical irritants on somatosensory receptors in the cat. *Neuropharmacology* 20: 191-198, 1981.
18. Jancso G, Kiraly E, Loo F, Such G, Nagy A. Selective degeneration of primary sensory neurones in the adult rat. *Neurosci. Lett.* 59: 209-214, 1985.
19. Heyman I, Rang HP. Depolarizing responses to capsaicin in a subpopulation of rat dorsal root ganglion cells. *Neurosci. Lett.* 56: 69-75, 1985.
20. Baccaglini PI, Hogan PG. Some rat sensory neurons in culture express characteristics of different pain sensory cells. *Proc. Natl. Acad. Sci., U.S.A.* 80: 594-598, 1983.
21. Wood JN, Winter J, James IF, Rang HP, Yeats J, Bevan S. Capsaicin-induced ion fluxes in dorsal root ganglion cells in culture. *J. Neurosci.* 8: 3208-3, 1988.
22. Szolcsanyi J. Capsaicin: hot new pharmacological tool. *Trends Neorosci.* 4: 495-497, 1984.
23. Moor RJ. Specific inhibition of mitochondrial Ca^{2+} transport by ruthenium red. *Biochem. Biophys. Res. Commun.* 42: 298-305, 1971.
24. Swanson PD, Anderson L, Stahl WL. Uptake of calcium ions by synaptosomes from rat brain. *Biochem. Biophys. Acta.* 356: 174-183, 1974.
25. Maggi CA, Santicioli P, Geppetti P, Parlani M, Astolfi M, Del Bianco E, Patacchini R, Giuliani S, Meli a. The effect of calcium free medium and nifedipine on the release of substance P-like immunoreactivity and contractions induced by capsaicin in the isolated guinea-pig and rat bladder. *Gen.Pharmocol.* 20: 445-456, 1989.
26. Amann R, Donnerer J, Lembeck F. Capaicin-induced stimulation of polymodal nociceptors is antagonized by ruthenium red independently of extracellular calcium. *Neurosci.* 32: 255-259, 1989.
27. Dickenson AH, Dray A, Hughes GA, WAlpole CSJ. The selective capsaicin antagonist capsazepine inhibits capsaicin-induced antinociception: electrophysiological studies in rodents. *Br. J. Pharmacol.* 102(suppl): 79, 1991.
28. Dickenson AH, Dray A. Selective antagonism of capsaicin by capsazepine: evidence for a spinal receptor site in capsaicin-induced antinociception. *Br. J. Pharmacol.* 104: 1045-1049, 1991.
29. Perkins MN, Campbell EA. Capsazepine reversal of the antinociceptive action of capsaicin in vivo. *Br. J. Pharmacol.* 107: 329-333, 1992.
30. Ohkubo T, Shibata M. The selective capsaicin antagonist capsazepine abolishes the antinociceptive action of eugenol and guaiacol. *J. Dent. Res.* 76(4):848-851, 1997.
31. Harper AA, Lawson SN. Conduction velocity is related to morphological cell type in rat dorsal root ganglion neurones. *J. Physiol. Lond.* 359: 31-46, 1985a.
32. Elliott AA, Elliott JR. Characterization of TTX-sensitive and TTX-resistant sodium currents in small cells from adult rat dorsal root ganglia. *J. Physiol. Lond.* 463: 39-56, 1993.
33. Cohen SA, Barchi RL. Voltage-dependent sodium channels. *Int. Rev. Cytol.*137: 55-103, 1993.
34. Akopian AN, Sivilotti L, Wood JN. A tetrodotoxin-resistant voltage-gated sodium channel expressed by sensory neurons. *Nature* 379: 257-262, 1996.
35. Caffrey JM, Eng DL, Black JA, Waxman SG, Kocsis JD. Three types of sodium channels in adult rat dorsal root ganglion neurons. *Brain Res.* 592: 283-297, 1992.
36. Kostyuk PG, Veselovsky NS, Fedulova SA, Tsyndrenko A Y. Ionic currents in the somatic membrane of rat dorsal root ganglion neurons - I. Sodium currents. *Neuroscience* 6: 2423-2430, 1981.
37. McLean MJ, Bennet PB, Thomas RM. Subtypes of dorsal root ganglion neurons based on different inward currents as measured by whole cell voltage clamp. *Molec. Cell. Biochem.* 80: 94-107, 1988.
38. Ogata N, Tatebayashi H. Ontogenic development of the TTX-sensitive and TTX-insensitive Na^+ channels in neurons of the rat dorsal root ganglia. *Dev. Brain Res.* 65: 93-100, 1992a.
39. Puil E, Gimbarzevsky B, Miura RM. Quantification of membrane properties of trigeminal root gangion neurons in guinea pigs. *J. Neurophysiol.* 55: 995-1016, 1986.
40. Schwartz A, Palti Y, Meiri H. Structural and developmental differences between three types of Na channels in dorsal root gangion cells of newborn rats. *J. Membr. Biol.* 116: 117-128, 1990.
41. Bossu JL, Feltz A. Patch-clamp study of the tetrodotoxin-resistant sodium current in group C sensory neurones. *Neurosci. Lett.* 51: 241-246, 1984.
42. Ogata N, Tatebayashi H. Slow inactivation of tetrodotoxin-insensitive Na+ channels in neurons of rat dorsal root ganglia. *J. Membr. Biol.* 129: 71-80, 1992b.
43. Cesare P, McNaughton P. Peripheral pain mechanisms. *Curr. Opin. Neurobiol.* 7: 493-499, 1997.
44. Reuter H. A variety of calcium channels. *Nature* 316: 391, 1985.
45. Tsien RW. Calcium currents in heart cells and neurons. *In Neuromodulation*, ed. Kaczmarek L. K. and Levitan I. B. Oxford: Oxford University Press. 1986.
46. Hagiwara S, Ozawa S, Sand O. Voltage-clamp analysis of two inward current mechanisms in the egg cell membrane of a star fish. *J. Gen. Physiol.* 65: 617-644, 1975.
47. Llinas R, Sugimori M. Electrophysiological properties of in vitro Purkinje cell somata in mammalian cerebellar slices. *J. Physiol.* 305: 171-195, 1980.
48. Scroggs RS, Fox AP. Distribution of dihydropyridine and ?-conotoxin-sensitive calcium currents in acutely isolated rat and frog sensory neuron somata: diameter-dependent `L channel expression in frog. *J. Neurosci.* 11: 1334-1346, 1991
49. Scroggs RS, Fox AP. Calcium current variation between acutely isolated adult rat dorsal root ganglion neurons of different size. *J. Physiol. Lond.* 445: 639-658, 1992.
50. Fox AP, Nowycky MC, Tsien RW. Kinetic and pharmacological properties distinguishing three types of calcium currents in chick sensory neurones. *J. Physiol. Lond.* 394: 149-172, 1987.
51. Mintz IM, Bean BP. Block of calcium channels in rat neu-

rons by synthetic ?-Aga-IVA. *Neuropharmacol.* 32: 1161-1169, 1992.
52. Glass RL, Zander HA. Pulp healing. *J. Dent. Res.* 28: 97-107, 1949.
53. Kozam G, Newark NJ. The effect of eugenol on nerve transmission. *Oral Surg.* 44: 799-805, 1977.
54. Dewhirlst FE, Goodson JM. Prostaglandin synthetase inhibition by eugenol, guaiacol and other dental medicaments. *J. Dent. Res.* 53(suppl): 104, 1974.
55. Hume W, Veaco N, Becker R. Concentration-dependent depression of cellular respiration by eugenol. *J. Dent. Res.* 61: 202, 1982.
56. Brodin P, Roed A. Effects of eugenol on rat phrenic nerve and phrenic nerve-diaphragm preparations. *Archs Oral Biol.* 29: 611-615, 1984.
57. Veaco N, Becker R, Hume W. Movement of 3H-eugenol through dentin. *J. Dent. Res.* 61:203, 1982.
58. Ritchie JM. A pharmacological approach to the structure of sodium channels in myelinated axons. *A. Rev. Neurol. Sci.* 2: 341-362, 1979.
59. Taylor RE. Effect of procaine on electrical properties of squid axon membrane. *Am. J. Physiol.* 196: 1071-1078, 1959.
60. Craft RM, Porreca F. Treatment parameters of desensitization to capsaicin. *Life Sci.* 57: 1767-1775, 1992.
61. Bevan S, Szolcsanyi J. Sensory neuron-specific actions of capsaicin: mechanisms and applications. *Trends Pharmacol. Sci.* 11: 330-333, 1990.
62. Bleakman D, Brorson JR, Miller RJ. The effect of capsaicin on voltage-gated calcium currents and calcium signals in cultured dosal root ganglion cells. *Br. J. Pharmacol.* 101: 423-431, 1990
63. Miller RJ. Multiple channels and neuronal function. *Science* 235: 46-52, 1987.