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Effects of Pain-Inducing Agents on Voltage Dependent Calcium Currents in the Trigeminal Ganglion

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Trigeminal ganglion (TG) neurons are primary sensory neurons involved in the transmission of orofacial sensation, including pain. It is known that intracellular free calcium concentration plays a critical role in neurotransmitter release. In the present study, we examined the effects of pain-inducing agents, bradykinin and histamine, on voltage dependent Ca^{2+} current (I_{Ca}) and compared these with the effects of the analgesic agent, eugenol. I_{Ca} was measured using a whole-cell patch clamp method. Bradykinin (10 μM) inhibited I_{Ca} in TG neurons. Maximum inward I_{Ca} was reduced by 26% by bradykinin compared with the controls (n=13, $p<0.05$). Decreased I_{Ca} induced by bradykinin did not recover to the prestimulus level after washing out bradykinin. In contrast, histamine (100 μM) increased I_{Ca} by 25% compared with controls (n=7, $p<0.05$) in an irreversible manner. Eugenol (250 μM), commonly used to produce analgesia in dental clinics, decreased I_{Ca} to 22.6% (n=9, $p<0.05$) and this effect on the I_{Ca} was reversible. Although the effect of eugenol on the I_{Ca} was consistent, the effects of the two pain-inducing agents, bradykinin and histamine, were different. Our results suggest that the modulation of I_{Ca} in TG neurons is not a decisive factor underlying the pain mechanism evoked by pain-inducing agents.

Keywords: trigeminal ganglion (TG), voltage dependent Ca^{2+} current (I_{Ca}), bradykinin, histamine, eugenol

Introduction

Tissue damage results in the local release of various biochemical species which mediate the inflammatory process. Bradykinin and histamine activate nociceptors and are directly involved in the pain producing process. Bradykinin, one of the important inflammatory mediators, stimulates nociceptive nerve terminals and induces the sensation of pain (Kumazawa and Mizumura, 1980); Manning *et al.*, 1991). Two types of bradykinin receptor, B1 and B2, have been defined pharmacologically (Farmer and Burch, 1992). The physiological significance of the B1 receptor is as yet unclear. In contrast, the B2 receptor is much better characterized, and accounts for the majority of the pharmacological effects of bradykinin. Histamine is released from mast cells in response to substance P, which is released from nociceptor terminals. It activates sensory neurons and evokes the sensations of itch and pain (Broadbent, 1955). It is believed that low concentrations of histamine induce itch and that higher concentrations cause pain (Simone *et al.*, 1991). However, the mechanisms by which histamine exerts its effects remain unknown.

There are various ion channels in the trigeminal ganglion (TG) neurons, including K^+ conductance (Puil *et al.*, 1988, 1989). In these neurons, Na^+ and Ca^{2+} contribute to the formation of tetrodotoxin-resistant long-duration action potentials. It has been suggested that the slow component of the spike, recorded in the presence of TTX, might be mediated by Ca^{2+} (Hsiung and Puil, 1990). Nonselective cation channels in TG neurons were reported to be activated by capsaicin (Liu and Simon, 1994, 1996). Studies on sensory neurons in culture (Burgess *et al.*, 1989; McGehee *et al.*, 1992) show that the excitatory effect of bradykinin is associated with an inward current and an increase in the membrane conductance mainly with respect to sodium ions. However, little is known about the role of the voltage dependent Ca^{2+} channel (I_{Ca}) in TG neuron pain transmission. In this study, we examined the effects of the pain-inducing agents, bradykinin and histamine, on I_{Ca}. The effect of eugenol, an analgesic, on the I_{Ca} was also examined and compared with the effects of the pain-inducing agents.

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Materials and Methods

Experimental solutions and reagents

Cold Ca^{2+}/Mg^{2+}-free phosphate buffered saline (CMF-PBS) solution was composed of 137 mM NaCl, 5 mM KCl, 22 mM KH_2PO_4, 5.5 mM glucose, 59 mM sucrose, phenol red and antibiotics. The bath and pipette solutions used for electrophysiological recordings were designed to record Ca^{2+} currents and minimize other ion-channel currents. The pipette solution was comprised of 100 mM CsCl, 20 mM tetraethylammonium (TEA)-Cl, 0.1 mM $CaCl_2$, 2 mM Mg-ATP, 10 mM ethyleneglycol bis (β-aminoethyl ether) N,N, N',N'-tetraacetic acid (EGTA) and 40 mM N-2-hydroxyethylpiperazine-N-2-ethane sulfonic (HEPES) acid at a final pH of 7.3. Bath solution contained 120 mM choline-Cl, 10 mM $CaCl_2$, 2 mM $MgCl_2$ and 10 mM HEPES at a final pH of 7.3. The pH was adjusted with CsOH. Bradykinin, histamine, eugenol and type XI trypsin were purchased from Sigma (USA).

Isolation of trigeminal ganglion (TG) neurons

TG neurons were isolated according to a previously published method (Oh *et al.*, 1997). Briefly, after identifying the mandibular and ophthalmic/maxillary division, the TG neurons on both sides were isolated surgically and the peripheral and central nerve roots excised. The ganglia were then immersed in CMF-PBS solution (see above) oxygenated with 95% O_2: 5% CO_2. Using a sterile Pasteur pipette, the ganglia were transferred into a 15 ml centrifuge tube containing trypsin (1 mg/ml) dissolved in CMF-PBS solution and then incubated at 37°C in a shaking water bath for approximately 45 min. After enzymatic treatment, they were washed four times with CMF-PBS solution and suspended in 3 ml of Dulbecco's Modified Eagle's Medium (Sigma, USA) for trituration. Fetal bovine serum (10% final concentration) was then added and the cultures maintained in an incubator at 37°C equilibrated with 5% CO_2 for 1-2 hours to allow the isolated cells to settle and adhere to the Cell-Tak coated coverslip. Calcium currents were recorded within 12 h of such treatment.

Electrophysiological recordings of I_{Ca}

Coverslips with adherent TG neurons were transferred to a recording chamber containing bath solution (see above), which was mounted onto an inverted microscope (Olympus IMT-2, Japan). Electrodes were made from microcapillary glass tubes (Chase Instruments Co., USA) using two pulls on a vertical microelectrode puller (PP-83, Narishige, Japan). Their tip resistance was between 3 and 5 MΩ. Electrodes were placed onto the cell membrane with a micromanipulator (Burleigh, USA).

Voltage dependent Ca^{2+} current (I_{Ca}) was recorded by the conventional whole-cell patch clamp method (Hamill *et al.*, 1981) and currents were measured with an Axopatch 1C amplifier (Axon Instruments, USA) at room temperature (18-25°C). Cell diameter was defined as the mean of the major and minor axis of each cell body. Only small to intermediate-sized neurons (<50 μm) were used in this experiment. Currents were evoked by step depolarization to between -70 and +60 mV in 10 mV increments from a holding potential of -80 mV, filtered at 5 KHz, digitally sampled by analogue-to-digital converter (TL-1 DMA interface, Axon Instruments, USA), and analyzed with pClamp 6 software (Axon Instrument).

Results

Morphologically isolated TG neurons were round with no processes. Neurons with cytoplasm appearing bifringent under phase contrast microscopy were considered to be healthy. The I_{Ca} was recorded from neurons with diameters less than 50 μm. Two types of I_{Ca} were observed in TG neurons based on their activation thresholds, i.e., low-

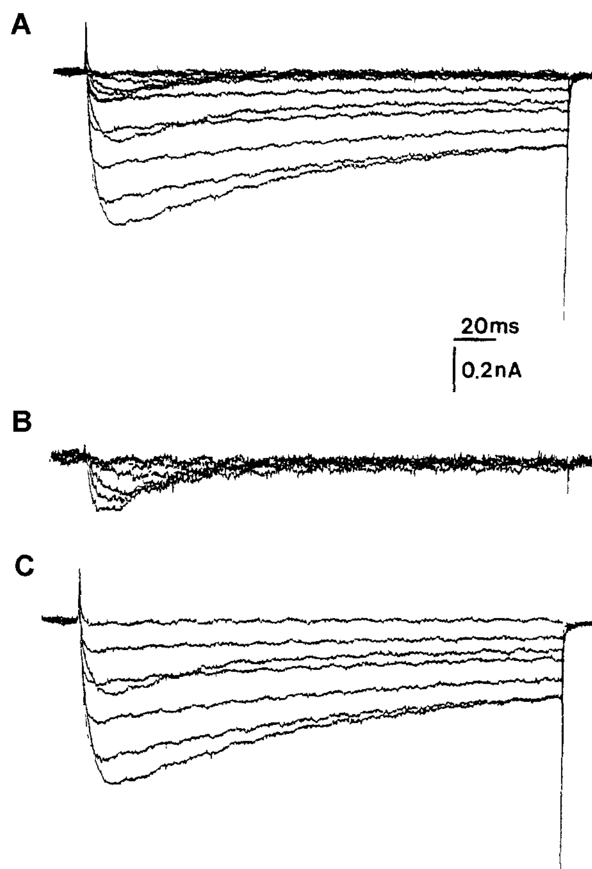

Fig. 1. Voltage dependent currents (I_{Ca}) in trigeminal ganglion (TG) neurons. (A) A typical profile of the voltage dependent currents (I_{Ca}) in trigeminal ganglion (TG) neurons. Both low voltage-activated (LVA) and high voltage-activated (HVA) currents were observed. (B) LVA Ca^{2+} currents activated from a membrane potential of -30 mV. The currents were of small amplitude and rapidly inactivated during the 40 msec time course. (C) HVA Ca^{2+} currents. Currents were of large amplitude and inactivated very slowly.

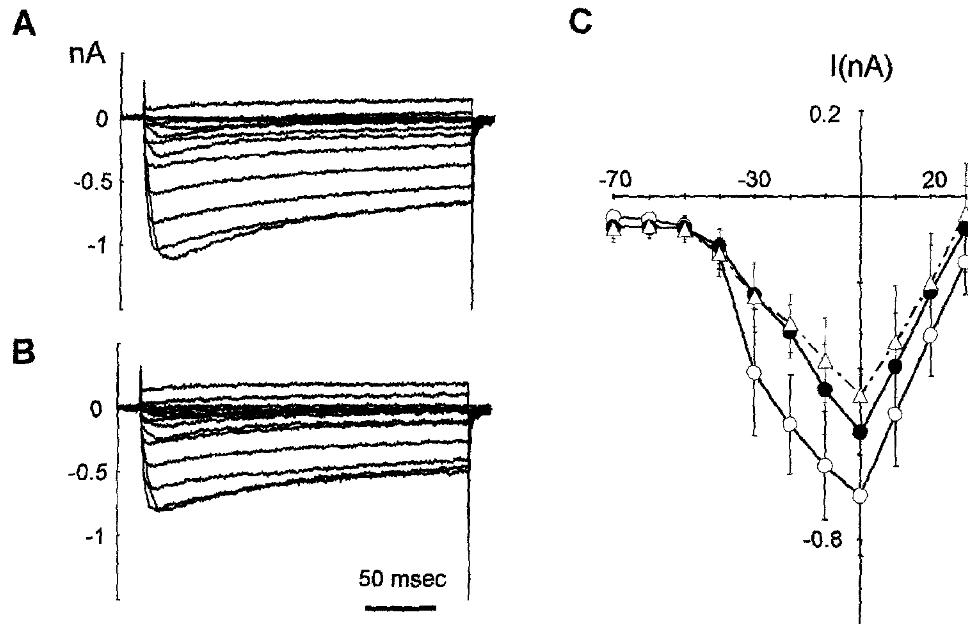

Fig. 2. The effect of bradykinin on the I_{Ca} in TG neurons. The effect of 10 μM of bradykinin on the I_{Ca} in TG neuron (n=13) before (A) and during (B) application. (C) Current-voltage relationships before (○), during (●) and after bradykinin washout (△). The average inhibition of maximal I_{Ca} by bradykinin was 26%. Horizontal axis means membrane potential (mV).

voltage-activated (LVA, Fig. 1B) or high-voltage-activated (HVA, Fig. 1C). LVA Ca^{2+} currents were activated at a membrane potential of -30 mV, which were inactivated during a 40 msec time course. HVA Ca^{2+} currents activated at a 0 mV command potential, and produced a current amplitude much larger than the LVA Ca^{2+} current and were inactivated very slowly. In the present study, we did not distinguish between the two types of I_{Ca} and examined the effects of bradykinin, histamine and eugenol on the mixed I_{Ca} (Fig. 1A).

The effect of bradykinin on I_{Ca} was variable. In five of 18 neurons, the I_{Ca} was increased after bradykinin stimulation. However, in most of the neurons (n=13), I_{Ca} was decreased by bradykinin addition to the bath solution. Figs 2A and 2B show typical Ca^{2+} currents from the 13 experiments before (A) and after (B) the application of 10 μM bradykinin. Both LVA and HVA Ca^{2+} currents were observed in these neurons. Currents from neurons shown in Fig. 2A and 2B were recorded using the leak subtraction protocol. Fig. 2C shows the mean current-voltage relationship before, during and after bradykinin washout. In the control bath solution, the inward current was activated from a membrane potential of -30 mV and reached a peak at membrane potential of 0 mV. In the presence of ten micromolar bradykinin, all currents above a membrane potential of -30 mV were reduced. Bradykinin significantly reduced the peak inward I_{Ca} from 768.1±105.5 pA (n=13) to 575.5±99.3 pA (n=13) ($p<0.05$ by paired t-test). The average inhibition of I_{Ca} by bradykinin was 26%. This decrease in I_{Ca} did not recover to the prestimulus level by washing out the bradykinin (data not shown, n=8, $p>0.1$).

In contrast to the effect of bradykinin, the I_{Ca} of most of the neurons was increased by exposure to 100 μM histamine (n=6). However, the I_{Ca} in three neurons decreased with histamine exposure; there was no change in the I_{Ca} of the other two neurons. Fig. 3A and Fig. 3B show typical Ca^{2+} currents before and after application of histamine for an average six experiments before and after application of histamine. In the control bath solution (Fig. 3A), the inward current was activated from a membrane potential of -30 mV. In the presence of histamine (Fig. 3B), the currents recorded at membrane potentials of -30 and -20 mV increased significantly. Fig. 3C shows the mean current-voltage relationship of six experiments. The maximal inward I_{Ca} in the membrane potential range between -30 and -10 mV significantly increased to -769.2±63.8 pA (n=6) from -617± 105.6 pA (n=6) ($p<0.05$, by paired t-test). The average increase of I_{Ca} by histamine was 25%, which did not recover to the prestimulus level by washing out the histamine (data not shown, n=6, $p>0.1$).

We then examined the effect of eugenol on the I_{Ca}. Eugenol decreased the I_{Ca} consistently in all the neurons we studied (n=9). Fig. 4A shows a typical I_{Ca} of nine experiments before, during and after washout of 250 μM eugenol. The I_{Ca} was recorded at a command potential of 0 mV stepped from a -80 mV holding potential and currents were not subtracted. The inward current at a membrane potential of 0 mV was reduced to -645 pA by eugenol (Fig. 4A) from the -1360 pA in the control bath solution (Fig. 4A). Application of 250 μM eugenol decreased the I_{Ca} by 53% and this decreased I_{Ca} recovered to 69% of the prestimulus level by washing out the eugenol (Fig. 4A). The

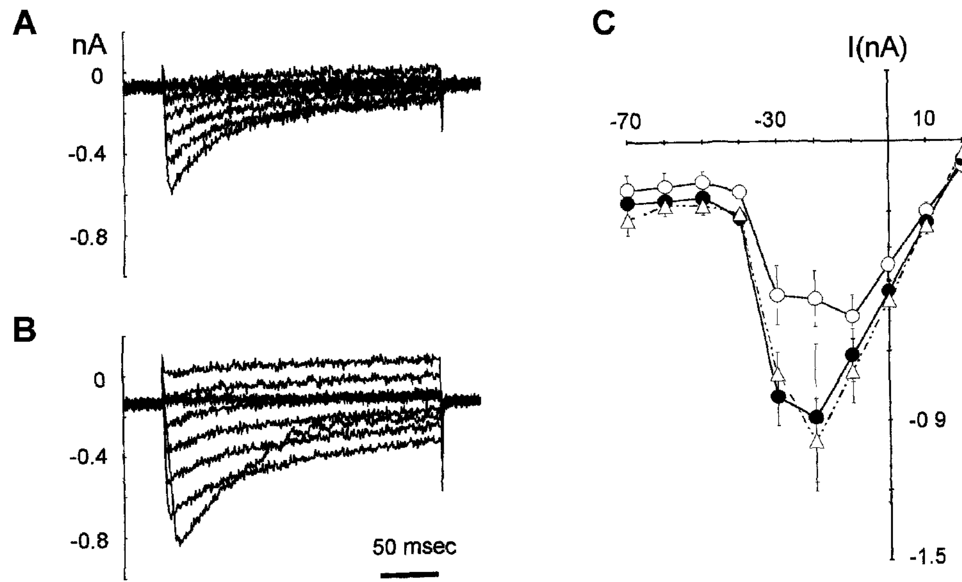

Fig. 3. The effect of histamine on the I_{Ca} in TG neurons. The effect of 100 μM histamine on the I_{Ca} in the TG neuron (n=6) before (A) and during (B) application. (C) Current-voltage relationships before (○), during (●) and after (△) histamine washout. The average increase of maximal ICa by histamine was 25%.

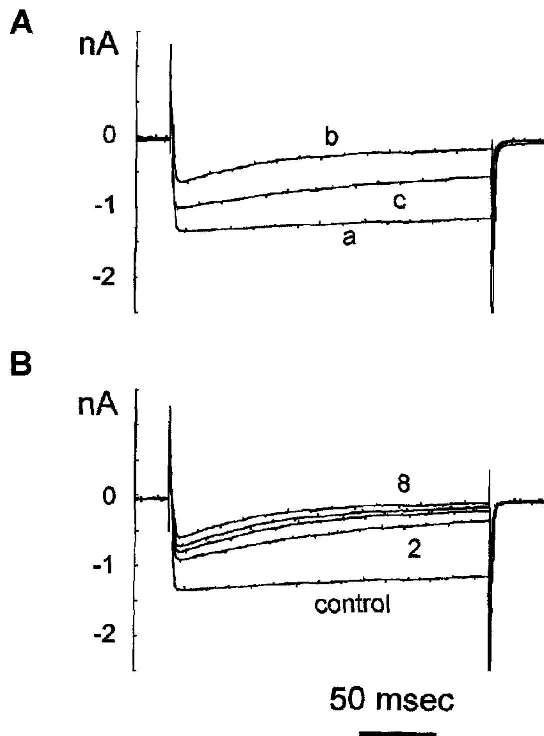

Fig. 4. The effect of eugenol on the I_{Ca} in TG neurons. (A) A typical I_{Ca} of nine experiments before (a) and during (b) application and after washout (c) of 250 μM eugenol in a TG neuron. I_{Ca} was recorded at a command potential of 0 mV stepped from a -80 mV holding potential. Application of 250 μM eugenol decreased I_{Ca} by 53% and this decreased I_{Ca} recovered to 69% of the prestimulus level by eugenol washout. (B) The effect of 250 μM eugenol on I_{Ca} after 2, 4, 6 and 8 min after application of eugenol.

average inhibition of I_{Ca} was 22.6±3.9% (n=9) in the range between 0.2 μM and 250 μM. In contrast to the results of bradykinin and histamine, the effect of eugenol on I_{Ca} was reversible (n=6). Fig. 4B shows a delayed eugenol effect on I_{Ca} in TG neurons. Maximal inhibition of I_{Ca} was observed at 6±1.2 min (n=9) after eugenol application.

Discussion

Voltage-dependent Ca^{2+} currents (I_{Ca}) in sensory neurons are well defined on the basis of their electrical properties and pharmacology (Nowychy *et al.*, 1985; Swandulla and Armstrong, 1988; Fox *et al.*, 1987; Mintz and Bean, 1993; Dichot *et al.*, 1995). In the present study, HVA and LVA-type currents were also observed in TG neurons. We investigated the effects of bradykinin, histamine and eugenol on mixed Ca^{2+} currents.

In nodose ganglion cells, bradykinin produced a different kind of excitatory effect associated with the inhibition of a long-lasting spike after hyperpolarization (Weinreich 1986; Weinreich and Wonderlin 1987). Studies on sensory neurons in culture (Burgess *et al.*, 1989; McGehee *et al.*, 1992) show that the excitatory effect of bradykinin is associated with an inward current and an increase in membrane conductance, mainly to sodium ions. However, in the present study, bradykinin inhibited I_{Ca} currents in TG neurons. Although our result, i.e., a decrease of inward Ca^{2+} currents by bradykinin, is hard to reconcile with the ability of bradykinin to evoke neuropeptide release from sensory neurons, it is in agreement with previous results (Kusano and Gainer, 1993; Connor and Henderson, 1997). In contrast to bradykinin, histamine

increased I_{Ca} in our experiments. Histamine receptor activation increased intracellular free Ca^{2+} concentration in TG (Tani *et al.*, 1990), which supports our results. Activation of I_{Ca} induced by histamine might contribute to an increase of $[Ca^{2+}]_i$. The different effects on I_{Ca} by bradykinin and histamine are probably due to their different intracellular transduction mechanisms. The main biochemical pathway through which bradykinin acts involves the G protein-mediated activation of phospholipase C, which generates two intracellular second messengers, 1,4,5-inositol-trisphosphate (IP3) and diacylglycerol (DAG) (Thayer *et al.*, 1988; Burgess *et al.*, 1989; Gammon *et al.*, 1989). However, the effect of histamine on the intracellular transduction mechanism is not known. Furthermore, the release of histamine involves further complex interactions, for example, histamine releases both tachykinins and calcitonin gene-related peptide (CGRP) in rat sensory neurons (Saria *et al.* 1988; Vedder and Otten 1991).

Eugenol has been commonly used in dental clinics to produce analgesia in cases of toothache, pulpitis and dentin hyperalgesia. In the present study, eugenol decreased I_{Ca} consistently in all the neurons we studied. The onset time of eugenol on I_{Ca} was, however, very slow compared to the response to agents such as bradykinin or histamine (delay of 3-4 min). This presumably reflects the time-course of the biochemical events that follow receptor occupation, presumably via vanilloid receptors. In dorsal root ganglion (DRG) neurons, eugenol activated Ca^{2+} permeable currents (Ohkubo and Kitamura, 1997). Our results indicate that I_{Ca} in TG neurons is modulated both by pain-inducing or analgesic agents, which could substantially contribute to the membrane potential and the intracellular concentration of free Ca^{2+}($[Ca^{2+}]_i$). However, the different effects of bradykinin and histamine on the I_{Ca} suggest that it is unlikely that Ca^{2+} influx via I_{Ca} is the only way of increasing $[Ca^{2+}]_i$. We cannot rule out the possibility of $[Ca^{2+}]_i$ increase via other pathways, e.g., involving a release of Ca^{2+} from the intracellular Ca^{2+} stores or Ca^{2+} influx via non-selective cation channels, which could override the decreased I_{Ca} induced by bradykinin.

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