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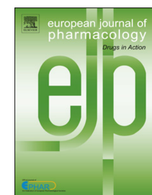

Neuropharmacology and analgesia

Carvacrol modulates voltage-gated sodium channels kinetics in dorsal root ganglia

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ABSTRACT

Recent studies have shown that many of plant-derived compounds interact with specific ion channels and thereby modulate many sensing mechanisms, such as nociception. The monoterpenoid carvacrol (5-isopropyl-2-methylphenol) has an anti-nociceptive effect related to a reduction in neuronal excitability and voltage-gated Na^+ channels (Na_V) inhibition in peripheral neurons. However, the detailed mechanisms of carvacrol-induced inhibition of neuronal Na_V remain elusive. This study explores the interaction between carvacrol and Na_V in isolated dorsal root ganglia neurons. Carvacrol reduced the total voltage-gated Na^+ current and tetrodotoxin-resistant (TTX-R) Na^+ current component in a concentration-dependent manner. Carvacrol accelerates current inactivation and induced a negative-shift in voltage-dependence of steady-state fast inactivation in total and TTX-R Na^+ current. Furthermore, carvacrol slowed the recovery from inactivation. Carvacrol provoked a leftward shift in both the voltage-dependence of steady-state inactivation and activation of the TTX-R Na^+ current component. In addition, carvacrol-induced inhibition of TTX-R Na^+ current was enhanced by an increase in stimulation frequency and when neurons were pre-conditioned with long depolarization pulse (5 s at −50 mV). Taken all results together, we herein demonstrated that carvacrol affects Na_V gating properties. The present findings would help to explain the mechanisms underlying the analgesic activity of carvacrol.
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1. Introduction

Throughout human history, natural products have had an essential role in the treatment of a number of diseases (Chang and Keasling, 2006; Koehn and Carter, 2005). Although the benefits are sometimes obvious, traditional or herbal medicine is regarded with skepticism, because the mechanism through which plant compounds exert their powers are largely elusive. Recent studies have shown however that many of these plant compounds interact with specific ion channels and thereby modulate the sensing mechanism of the human body (Alvarez-Collazo et al., 2014; Santos-Nascimento et al., 2015; Straub et al., 2013).

Voltage-gated Na^+ channels (Na_V) underlie the generation of action potentials that are extremely important for electrical communication between excitable cells. Modulation of Na_V exerts important impact on the control of neuronal excitability (Ahn et al., 2007). Importantly, Dorsal root ganglia (DRG) neurons were chosen to pursue this study because their cell bodies or afferent fibers are carriers of sensory information. With regard to cell size, cultured mammalian DRG neurons have been classified into two major groups (1) large neurons with short-lasting action potentials predominantly expressing fast tetrodotoxin-sensitive Na^+ currents (TTX-S); and (2) small neurons with long-lasting action potentials, expressing a combination of fast TTX-S and slow TTX-resistant (TTX-R) Na^+ currents (Cummins et al., 2007; Dib-Hajj et al., 2010; Roy and Narahashi, 1992). Recently, single-cell analysis of Na^+ channel transcripts indicated that TTX-S and TTX-R are differentially expressed in large and small sensory neurons and the authors provided evidence that Na_V 1.8 is highly expressed in a subpopulation of large myelinated DRG neurons (Ho and O'Leary, 2011). These results support previous findings that have identified Na_V 1.8 expression in large-diameter DRG neurons by immunolabelling (Amaya et al., 2000; Djouhri et al., 2003)

Bioprospection of new drugs that could be useful in the treatment of pain is an increasingly active research field. Carvacrol, 5-isopropyl-2-methylphenol, is an organic compound naturally occurring in essential oils of many plants. It is well known for its

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wide pharmacological profile, especially analgesic (Guimarães et al., 2010). Previous studies have demonstrated that carvacrol elicited a significant inhibition of neuronal action potential (Gonçalves et al., 2010; Joca et al., 2012).

Although previous results have indicated that carvacrol inhibits voltage-dependent Na^+ currents, the mechanisms underlying its blocking effects remain elusive. In this study, we used whole-cell patch-clamp to further investigate the effects of carvacrol on Na^+ channels. In addition, we studied the effects of carvacrol on TTX-R Na^+ currents carried by Na_V 1.8 channels from DRG neurons.

2. Materials and methods

2.1. Isolation and cell culture of rat DRG neurons

DRG neurons were obtained from the lumbar segments of 10 to 14-w old male Wistar rats (220–250 g body weight) using an enzymatic dissociation procedure as described previously (Joca et al., 2012; Moraes et al., 2011). Rats were rendered unconscious by exposure to CO_2 and decapitated. Lumbar DRG were rapidly dissected and placed in Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution (HBSS). After carefully removing the surrounding connective tissues, each ganglion was incubated for 75 min, in a water bath at 37 °C, in HBSS containing type 1 collagenase 1 mg/ml (Sigma Chemical Co., St. Louis, MO, United States) and further incubated for 15 min at 37 °C in HBSS containing 2.5 mg/ml trypsin (Sigma Chemical Co.). The enzyme digested ganglia were mechanically agitated using a firepolished Pasteur pipette to disperse the neurons. The dissociated neurons thus obtained were washed twice with Dulbecco's Modified Eagle's Medium (Sigma Chemical Co.) supplemented with 10% fetal bovine serum and 1% penicillin (Cultilab, Brazil). The neurons were then plated onto poly-D-lysine (0,1%, Sigma Chemical Co.)-coated rectangular glass coverslips and were incubated at 37 °C for 12–48 h in a humidified atmosphere of 95% air plus 5% CO_2 prior to the experiments. DRG neurons that appeared practically spherical without neuronal processes were used for experiments. All experimental procedures were reviewed and approved by the institutional ethics committee of Universidade Federal da Paraíba (CEUA-UFPB).

2.2. Electrophysiology

Sodium currents were recorded at room temperature (24–28 °C) in the whole-cell mode of the patch-clamp technique (Hamill et al., 1981) using a HEKA EPC-9/2 amplifier (HEKA Instruments, Germany). Whole-cell currents were acquired at 20 kHz and filtered at 3 kHz. Pulse generation and data acquisition were performed on a Windows-based computer using the Patchmaster program (version 2.7; HEKA, Lambrecht/Pfalz, Germany). Bath solution contained (in mM): NaCl 140, KCl 5, $CaCl_2$ 1.8, $MgCl_2$ 0.5, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES) 5, and glucose 5 with pH adjusted to 7.4. Recording electrodes (1–2 MΩ) were fabricated from capillary glass using a HEKA PIP6 pipette puller (HEKA, Lambrecht/Pfalz, Germany). The standard pipette solution contained (in mM): NaCl 10, CsCl 100, HEPES 10, ethylene glycol tetraacetic acid 11, tetraethylammonium-Cl 10, $MgCl_2$ 5, and pH adjusted to 7.2 with CsOH. Series resistance errors were compensated using 60–85% series resistance compensation. DRG neurons on glass coverslips were transferred into a recording chamber containing standard bathing solution. After establishing the whole-cell configuration, the bath solution was changed to one containing (in mM): NaCl 40, KCl 3, HEPES 10, tetraethylammonium-Cl 20, $CaCl_2$ 1, $MgCl_2$ 1, $CdCl_2$ 0.1, Choline-Cl 70, and glucose 10 with pH adjusted to 7.4. The extracellular Na^+ concentration was maintained at 40 mM for all experiments to reduce the size of Na^+ current and thus improve voltage-clamp conditions. Cd^{2+} (100 μM) was routinely included in the bath solution to block Ca^{2+} channels. Although sub-milimolar concentrations of Cd^{2+} have been reported to block tetrodotoxin-resistant Na^+ channels (Ikeda and Schofield, 1987), its use in the present study does not affect our conclusions because even in the presence of Cd^{2+} the size of tetrodotoxin-resistant Na^+ currents were sufficiently large to allow careful analysis (very large signal-to-noise ratio). In a series of experiments, currents in DRG neurons were measured in the presence (300 nM) of tetrodotoxin.

DRG neurons were held at −80 mV for 5 min before initiating the experimental stimulation protocols to allow cell dialysis to complete (Pusch and Neher, 1988). The DRG neuron under examination was continuously perfused via a large-bore perfusion pipette positioned with a mechanical micromanipulator in its vicinity. External test solutions without (control) or with carvacrol were changed by an electric command to a micro-solenoid valve (The Lee Co., Essex, CT, USA) that controlled the bath perfusion.

Peak Na^+ currents were measured using 100 ms pulses to between −120 mV and 50 mV every 5 s from a holding potential of −80 mV. The peak current was normalized for cell capacitance and plotted against voltage to generate peak current density–voltage relationships. Whole-cell conductance was calculated from the peak current amplitude using the equation $(E_m - E_{rev})*G_{max}/(1+\hat{e}((V_m - E_m)/k_a)))$. E_{rev} is the estimated Na^+ current reversal potential, and then normalized to the maximal conductance recorded between 5 and 25 mV. The normalized conductance as a function of voltage curves was fit with the Boltzmann function, $(G - G_{max})/(1+\hat{e}(((E_m - V_a)/k_a)))$ determine the voltage for half-maximal channel activation (V_a) and slope factor (k_a). The voltage dependence of channel availability was assessed after a 300 ms prepulse to various potentials followed by 100 ms pulse to 0 mV, the voltage at which peak Na^+ currents was measured. The normalized current was plotted against the voltage, and steady-state channel availability curves were fit with Boltzmann function "$1/(1+\hat{e}(((E_m - V_h)/k_h)))$", to determine the voltage for half-maximal channel inactivation (V_h) and slope factor (k_h).

2.3. Chemicals and solutions

Carvacrol was obtained from Sigma Chemical Co. A 10 mM stock solution was made up in dimethylsulfoxide (DMSO). All solutions were prepared fresh daily. In a series of control experiments, 10 min application of DMSO at 0.2% (v/v) had negligible effects on the functional properties of TTX-S and TTX-R Na^+ currents (data not shown).

2.4. Data analysis

All data are expressed as means ± S.E.M., where n indicates the number of experiments. The unpaired and paired Student's t-test were used when appropriate, $P < 0.05$ is considered to indicate the statistically significant difference. Mathematical curve fitting was accomplished using SigmaPlot (11.0, Systat Software). All curve fitting routines were performed using non-linear regression analysis (Levenberg–Marquadt algorithm).

3. Results

To test the hypothesis that ion channel antagonism may underlie the anti-nociceptive effects of carvacrol we investigated the blocking effect of carvacrol on voltage-gated Na^+ channels in isolated rat DRG neurons. We focused our attention on large DRG neurons (estimated diameter ≥ 30 μm based on membrane capacitance measurements) that has been reported to express TTX-S and TTX-R voltage-dependent Na^+ currents (Caffrey et al., 1992).

Carvacrol blocks all voltage-dependent Na^+ currents in a concentration-dependent fashion (10–2000 μM, n=50 cells) in

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