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Research article

Effects of 1,8-cineole on Na^+ currents of dissociated superior cervical ganglia neurons

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HIGHLIGHTS

- 1,8-Cineole promote partial blockade of Na^+ current of dissociated SCG neurons.
- Alterations of Na^+ current kinetics.
- Deviation of activation and inactivation curves toward hyperpolarized potentials.
- Partial explanation for changes on excitability of sciatic nerve and SCG neurons.

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ABSTRACT

1,8-Cineole is a terpenoid present in many essential oil of plants with several pharmacological and biological effects, including antinociceptive, smooth muscle relaxant and ion channel activation. Also, 1,8-cineole blocked action potentials, reducing excitability of peripheral neurons. The objective of this work was to investigate effects of 1,8-cineole on Na^+ currents (I_{Na}^+) in dissociated superior cervical ganglion neurons (SCG). Wistar rats of both sexes were used (10–12 weeks old, 200–300 g). SCG's were dissected and neurons were enzymatically treated. To study 1,8-cineole effect on I_{Na}^+, the patch-clamp technique in whole-cell mode was employed. 1,8-Cineole (6.0 mM) partially blocked I_{Na}^+ in SCG neurons. The effect stabilized within ~150 s and there was a partial recovery of I_{Na}^+ after washout. Current density was reduced from −105.8 to −83.7 pA/pF, corresponding to a decrease to ~20% of control. 1,8-Cineole also reduced the time-to-peak of I_{Na}^+ activation and the amplitude and decay time constants of I_{Na}^+ inactivation. Current–voltage plots revealed that 1,8-cineole left-shifted the $V_{1/2}$ of both activation and inactivation curves by ~10 and ~20 mV, respectively. In conclusion, we demonstrate that 1,8-cineole directly affects Na^+ channels of the SCG by modifying several gating parameters that are likely to be the major cause of excitability blockade.

1. Introduction

1,8-Cineole is a terpenoid present in the essential oils of plants of several genuses, including *Eucalyptus* [1–4] and *Croton* [5,6]. Several pharmacological and biological effects of 1,8-cineole have been described, including smooth muscle relaxant [4,7], hypotensive, bradycardic [8], anti-inflammatory and antinociceptive [9] activities. 1,8-Cineole is also reported to activate human transient receptor potential cation channel (TRP) M8 (TRPM8) and block TRPA1 channels [10].

Regarding effects on the nervous system, 1,8-cineole blocked the excitability of sciatic nerve [5] and superior cervical ganglia (SCG) neurons [11]. Specifically, 1,8-cineole depolarized the resting potential and blocked action potentials (AP) in SGC neurons [11]. This AP blockade was partially reversed by artificially (intracellular current injection) repositioning resting potential to control voltages. For this reason, this blockade was interpreted as being partially due to an indirect action on Na^+ channels (I_{Na}^+ inactivation

Abbreviations: SCG, superior cervical ganglia; TRPM8, transient receptor potential cation channel subfamily M member 8; TRPA1, transient receptor potential cation channel subfamily A member 1; I_{Na}^+, Na^+ current; AP, action potential.

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via depolarization). However, since this reversal through resting potential handling was only partial and since several terpene and terpenoid molecules directly act on Na^+ channels [12–15], we hypothesized that 1,8-cineole may also direct regulate Na^+ channels, an effect that contributes to its excitability blocking effect. This study aims to investigate this hypothesis.

2. Material and methods

2.1. Animal procedures

All animal procedures were first reviewed and approved by the animal ethics committee of State University of Ceará, protocol number 6379067-0. All efforts were made to minimize animal suffering. The animals employed (Wistar rats of both sexes, 200–300 g body mass) were kept in controlled temperature (22–26 °C) and light-dark cycle (12/12 h). Animals were sacrificed in a CO_2 chamber, and SCGs were dissected and cleaned in cold (4 °C) Ca^{2+}/Mg^{2+}-free Hank's balanced salt solution (HBSS). The dissociation and maintenance of SCG's neurons was adapted and done as described by Ikeda (2004) [16]. The cells were used within 12 h after dissociation.

2.2. Solution and drugs

The HBSS solution contained (mM): NaCl 137.93, $NaHCO_3$ 4.0, Na_2HPO_4 0.3, KCl 5.33, KH_2PO_4 0.44, glucose 5.6, pH adjusted to 7.4 with NaOH. For patch-clamp studies, we first maintained the coverslips with cells in a bath solution before $I_{Na}{}^+$ register. The composition of the bath solution was (mM): NaCl 140, KCl 5.0, $CaCl_2$ 1.8, $MgCl_2$ 0.5, HEPES 5.0, and glucose 5.0. To study the I_{Na+} in SCG we perfused neurons with an external solution modified from Cummins et al. [17] whose composition was (mM): NaCl 40, choline chloride 70, KCl 3.0, $CaCl_2$ 1.0, $MgCl_2$ 1.0, tetraethylammonium chloride 20, $CdCl_2$ 0.1, HEPES 10, and glucose 10. The pH of all solutions was adjusted to 7.4 with HCl, except for the modified external solution, which was adjusted with tetraethylammonium hydroxide. The internal solution contained (mM): NaCl 10, CsCl 100, HEPES 10, ethylene glycol tetraacetic acid 11, tetraethylammonium chloride 10, and $MgCl_2$ 5.0, pH adjusted to 7.2 with CsOH. Choline was used as a non-permeant monovalent cation in partial substitution of external Na^+ in order to improve the quality of voltage clamp data and Cs^+ and tetraethylammonium were used to block K^+ channels, and Cd^{2+} was used to block Ca^{2+} channels. All experiments were carried out at room temperature (20–24 °C).

1,8-Cineole was daily dissolved in dimethylsulfoxide (DMSO) to prepare stock solutions. To provide the desired drug concentration, the stock solutions were added to the patch-clamp external solution with caution to maintain DMSO final concentration ≤0.2% v/v. All salts, drugs and enzymes were purchased from Sigma Chemical (St Louis, MO, USA), Reagen (Rio de Janeiro, RJ, Brazil) and Roche (Basel, Switzerland) and were of analytical grade.

2.3. Patch clamp recordings

Coverslips with dissociated SCG neurons were placed in a recording chamber on the stage of an inverted microscope (model Axiovert 200, Carl Zeiss Inc., USA) and perfused with bath solution. The recording electrodes and the procedures to achieve whole-cell configuration were as previously described [12,14]. Each neuron underwent at least 30 s of stable control recording (perfusion with external solution) followed by another period of exposure to 1,8-cineole containing solution to then exposure to drug-free. The neurons were voltage-clamped at −80 mV (holding potential) for all experimental manipulations. A 100 ms voltage step to 0 mV, 0.2 Hz interval, was employed to elicit $I_{Na}{}^+$. Capacitance and leakage subtraction were performed using a P/4 subtraction protocol.

Table 1
Na^+ current parameters of dissociated superior cervical ganglia neurons in control and 1,8-cineole conditions.

Parameter	Control	6 mM ClN
Peak $I_{Na}{}^+$ current (pA)	−3113 ± 327.6	−2431 ± 243.2*
Current density at 0 mV (pA/pF)	−106 ± 11.9	−84 ± 9.8*
Time-to-peak (ms)	0.6 ± 0.0	0.5 ± 0.0*
A_{slow} (pA)	−190 ± 46.5	−133 ± 37.0
τ_{slow} (ms)	3.8 ± 0.4	3.0 ± 0.3
A_{fast} (pA)	−2835 ± 283.3	−2158 ± 211.1#
τ_{fast} (ms)	0.6 ± 0.0	0.5 ± 0.0*

Data are reported as mean ± SE. The number of cells used were 16 for both groups (paired data). The symbols * and # indicate statistical diference compared to control group ($p < 0.05$, paired t-test and Wilcoxon signed rank test, respectively).

Series resistance compensation [70–80%] was routinely employed to reduce voltage error. The current was sampled at 40 kHz and low-pass filtered at 2 kHz, and data acquisition and storage were performed using computer acquisition hardware (Digidata 1440A model, Axon Instruments). To assess current kinetics, we measured time-to-peak for activation and inactivation decay time constants. Time-to-peak was determined by the time elapsed from beginning to the peak of $I_{Na}{}^+$. Inactivation of $I_{Na}{}^+$ was fitted with a double exponential function as follow

$$I_{Na}^{+}(t) = A_{fast} \times \exp\left(\frac{-t}{\tau_{fast}}\right) + A_{slow} \times \exp\left(\frac{-t}{\tau_{fast}}\right)$$

and functions parameters were denoted here as fast and slow inactivation amplitude (A_{fast} and A_{slow}) and time (τ_{fast} and τ_{slow}) constants.

2.4. Na^+ current activation, steady-state inactivation and whole-cell analysis

In order to record $I_{Na}{}^+$ steady-state activation and inactivation, a series of voltage pulse protocols were used. From holding potential, a 400 ms pre-pulse voltage step to −120 mV was employed to maximize the fraction of Na^+ channels in closed state. A current–voltage relationship (I–V plot) was obtained using an 80 ms depolarizing pulse from pre-pulse voltage step to +50 mV. At the end of depolarizing pulse, a 20 ms voltage step to 0 mV was set to investigate steady-state $I_{Na}{}^+$ inactivation. The absolute currents elicited by voltage steps were normalized by capacitance. For determination of steady-state activation and inactivation curves, the peak current was normalized by its maximum value and fitted by the Ohm–Boltzmann equation.

2.5. Statistical analysis

Data are reported as means ± SE with "n" indicating the number of experiments. Tests used to detect statistical significance ($p < 0.05$) were paired t-test for parametric data and Wilcoxon signed rank test for non-parametric data.

3. Results

Mean cell capacitance was 31 ± 1.5 pF, with access resistance of 4 ± 0.5 MΩ and leak current of −122 ± 27.6 pA ($n = 16$). Perfusion time with 1,8-cineole (6.0 mM) lasted until stabilization of the response (<150 s) to a depolarization pulse to 0 mV. These times and concentrations are similar to those previously demonstrated to block AP's in intact SCG neurons [11]. Values of $I_{Na}{}^+$ in control conditions are provided in Table 1. Vehicle (DMSO, <0.2% v/v) did not alter $I_{Na}{}^+$. 1,8-Cineole (6.0 mM) blocked $I_{Na}{}^+$, as shown in Fig. 1A and Table 1. In all cells, steady-state inhibition occurred within the exposure time (∼150 s, Fig. 1B). 1,8-Cineole reduced mean current