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Eucalyptol induces hyperexcitability and epileptiform activity in snail neurons by inhibiting potassium channels

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ABSTRACT

The effects of eucalyptol (1,8-cineole) were studied on the activity of central neurons of land snail *Caucasotachea atrolabiata*. Eucalyptol (3 mM) depolarized the membrane potential and increased the frequency of spontaneous activity in a time dependent and reversible manner. These effects were associated with suppression of afterhyperpolarization and significant reduction of amplitude and slope of rising and falling phases of action potentials. While the eucalyptol-induced suppression of action potential amplitude and rising slope were essentially dependent on membrane depolarization, its actions on repolarization slope and afterhyperpolarization were not affected by resetting the membrane potential close to the control value. These findings suggest an inhibitory action on the potassium channels that underlie repolarization and afterhyperpolarization. Eucalyptol also increased the frequency of driven action potentials but suppressed the post stimulus inhibitory period, indicating an inhibitory action on calcium-activated potassium channels.

A higher concentration of eucalyptol, 5 mM, reversibly changed the pattern of activity to burst firing associated with paroxysmal depolarization shift (PDS). Low doses of eucalyptol and potassium channel blockers, tetraethylammonium and 4-aminopyridine, synergistically acted to induce burst firing. At high concentration (30 mM), tetraethylammonium was able to induce burst firing and PDS. The sodium currents and ion channel phosphorylation by protein kinases A and C were not required for the eucalyptol-induced epileptiform activity, but calcium currents were essential for this action. Our findings show the excitatory and epileptogenic action of eucalyptol, which is most likely mediated through direct inhibitory action on potassium channels.

1. Introduction

Despite of the common assumption that natural substances are safe, they can have adverse effects when used inappropriately. Convulsion is one of the most hazardous symptoms that have been associated with indiscriminate usage of some plant essential oils (Burkhard et al., 1999). Many reports have described the convulsive properties of some essential oils, but the knowledge about the effective components and their mechanism(s) of action are usually incomplete.

Eucalyptol ($C_{10}H_{18}O$), known also as 1,8-cineole, is a monoterpenoid cyclic ether that constitutes the major component of *Eucalyptus* essential oil (Zhang et al., 2014). It is used as flavoring and fragrance in various products because of its fresh and pleasant smell, or spicy taste and cooling effect. It is widely used in many brands of toothpaste, mouthwash, shampoo and, as a food grade flavor ingredient, it can be found in the list of additives to cigarettes and even some food products. The effects of eucalyptol on transient receptor potential (TRP) and cyclic nucleotide-gated channels of olfactory and gustatory receptors provide a basis for its special taste and odor (Chen et al., 2006; Leffingwell, 2009). It has also a broad range of applications in therapeutics and especially used in the folk medicine as a remedy for respiratory problems because of its antiseptic, decongestant and expectorant properties (Juergens et al., 2003). Eucalyptol reduces inflammation and pain when applied topically (Ximenes et al., 2013). The agoniztic action of this compound on TRPM8 channels underlies its cooling action. The analgesic and anti-inflammatory effect of eucalyptol is possibly due to its inhibition of TRPA1 and desensitization of TRPV3 channels (Kolassa, 2013).

In addition to interaction with ion channels on the peripheral sensory cells, eucalyptol has been reported to show smooth muscle relaxant, hypotensive and bradycardic properties. It also

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reduces the excitability of rat sciatic nerve and superior cervical ganglion neurons (Lima-Accioly et al., 2006; Ferreira-da-Silva et al., 2009). On the other hand, it has been suggested that eucalyptol and some other oxygenated monoterpenoids, contributes to the convulsant action of several essential oils (Burkhard et al., 1999; Culić et al., 2009; Kolassa, 2013; Waldman 2011). The effect of eucalyptol on the central neurons that underlie its convulsive action may involve interaction with different ion channels, but these mechanisms have not been described yet. Snail neurons have the essential substrates to develop and sustain epileptiform activity that in many aspects resemble those that happening in vertebrate neurons. These neurons have often been used as model system both for studying the neurophysiological basis of epilepsy and also for primary screening of compounds with potential efficacy in inducing or preventing epileptiform activity (Altrup et al., 1992; Onozuka et al., 1991). In this work, intracellular recording techniques under current clamp conditions were carried out on snail neurons to determine the possible epileptogenic action of eucalyptol and the ionic mechanisms that may underlie this effect.

2. Material and method

2.1. Animals and preparations

Adult specimens of land snail *Caucasotachea atrolabiata* were collected from gardens in Babol, on the Caspian Sea coast, and kept under laboratory conditions. Animals were treated in accordance with the EC guidelines and the experimental protocols were reviewed and approved by the animal care committee of the Shiraz University. Snails were activated by wetting once or twice per week and fed with lettuce and carrot. All experiments were performed on neurons in the subesophageal ganglia. Animals were activated in cold water and the ganglionic mass with its main peripheral nerves and aorta was rapidly dissected out. The subesophageal ganglia were pinned by the nerve and edges of the connective tissue into a small (1 ml) Sylgard-grounded recording chamber (Dow Corning Midland, MI, USA) containing normal snail Ringer. To expose neurons, the overlying connective tissue was gently torn out using fine forcipes without any proteolytic enzyme pretreatment.

2.2. Intracellular recording

Recording electrodes were fabricated from thin-wall (outer diameter 1.5 mm, inner diameter 1.12 mm) borosilicate glass capillaries using a horizontal micropipette puller (P-97, Sutter Instrument Co., USA), filled with 3 M KCl and those with a resistance of 1–5 MΩ were used for recording. SEC-10LX amplifier (npi, Germany) was used to record the membrane potentials and to inject current under current clamp conditions. Data were digitized using a LIH 8+8 data acquisition interface (HEKA, Germany) and analyzed by Fitmaster software (HEKA, Germany). Neurons with stable resting membrane potentials (RMP) more negative than −38 mV were studied. The RMP was defined as the mean voltage during interspike intervals, excluding the decaying phase of afterhyperpolarization and the depolarizing ramp that precedes each action potential. Hyperpolarizing current steps (1–5 nA, 500 ms) were injected into the neurons and steady-state voltage changes from RMP were plotted against the injected currents. The R_{in} was calculated from a linear fit of the current–voltage plot. Spike amplitude was defined as the change in voltage from the RMP to the peak of spike. The amplitude of afterhyperpolarization was measured from the RMP to the peak negativity after a spike and the duration was measured as the time required declining to 80% of its peak value. Spike threshold was estimated by eye as the sharp inflection point at which the action potential began. The slope of rising phase was measured as the mean slope between the threshold potential and the peak of the action potential, and the slope of falling phase was measured as the slope between peak of action potential and peak of afterhyperpolarization.

2.3. Solutions and drugs

The normal snail Ringer solution contained (in mM): NaCl 80, KCl 4, $CaCl_2$ 10, $MgCl_2$ 5, glucose 10, HEPES 5; pH adjusted to 7.4 with TRISMA-base. The Na^+-free Ringer was prepared by substituting NaCl with equimolar quantities of Tris–HCl. Eucalyptol, tetraethyl ammonium (TEA), 4-aminopyridine (4-AP), dimethyl sulfoxide, nifedipine, N-[2-(p-Bromocinnamylamino) ethyl]-5-isoquinolinesulfonamide dihydrochloride (H-89) and chelerythrine were purchased from Sigma (St. Louis, MO, USA). Other chemicals were obtained from Merck (Darmstadt, Germany). Eucalyptol was prepared as 0.5 M stock in 60% ethanol and diluted (3–5 mM) daily in snail Ringer. Stock solutions of H-89 and chelerythrine were made in dimethyl sulfoxide and stored at −20 °C in single-use aliquots for direct bath application. Nifedipine (a blocker of L-type Ca^{2+} channel) was prepared in a stock solution of 10 mM in 95% ethanol and 1 M stock solutions of TEA and 4-AP were daily prepared in double distilled water. TEA-containing Ringer was prepared by replacing NaCl with equimolar amounts of TEA.

2.4. Statistical analysis

Data were presented as mean ± S.E.M. with n being the number of neurons on which the measurements were done. The statistical differences were analyzed with either paired *t*-tests, or repeated measures ANOVA followed by Bonferroni *post-hoc* tests. A value of $P < 0.05$ was considered as statistically significant.

3. Results

3.1. The effects of eucalyptol on neuronal firing, action potential characteristics and pattern of activity

All of the studied neurons showed rhythmic spontaneous firing in control condition. Extracellular application of eucalyptol (3 mM) increased the frequency of action potentials in a time dependent manner (Fig. 1A). This effect was along with membrane depolarization and modulatory actions on action potential characteristics, which has been summarized in Table 1. Especially, the excitatory effect of eucalyptol was associated with partial suppression of afterhyperpolarization and significant reduction of amplitude and the slope of rising and falling phases of action potentials (Fig. 1B). The effects of eucalyptol on firing frequency, RMP and action potential configurations showed recovery after 1–2 min washing with normal Ringer at a rate of approximately 3 ml/min (Table 1, Fig. 1B).

Since the effects of eucalyptol on the frequency and the waveform of action potentials were associated with a significant membrane depolarization, Pearson's correlation test was used to determine if correlations existed between RMP and R_{in}, action potential parameters and excitability. The RMP was not correlated with R_{in} but significant positive correlations between RMP (mean voltage of interspike interval) and firing frequency, action potential threshold and integral was detected. The action potential amplitude, rising and falling slope and the afterhyperpolarization duration and amplitude showed significant negative correlations with RMP (Table 2). The correlation found between RMP and some variables does not necessarily imply that RMP changes underlie