NeuroToxicology 60 (2017) 299–307

Contents lists available at ScienceDirect

NeuroToxicology

Full length article

Camphor elicits epileptiform discharges in snail neurons: The role of ion channels modulation

Jafar Vatanparast*, Fatemeh Andalib-Lari

Department of Biology, College of Science, Shiraz University, Shiraz, Iran

ARTICLE INFO

Article history:
Received 2 October 2015
Received in revised form 15 December 2015
Accepted 15 December 2015
Available online 21 December 2015

Keywords:
Camphor
Snail neuron
Action potential
Epileptiform activity
Ion channels

ABSTRACT

Generalized convulsion is one of the most prominent manifestations of camphor toxicity, but the mechanism underlying this effect has not been elucidated. Here, we examined the excitatory and epileptogenic action of camphor in snail neurons and studied the cellular and molecular mechanisms that are involved to these effects. The spontaneous activities of neurons from subesophageal ganglia of snail *Caucasotachea atrolabiata* were recorded using single-electrode current clamp under control condition and after exposure to different concentrations of camphor and ion channel blockers. Under control condition, the studied neurons showed regularly spaced spontaneous action potentials. Exposure to low concentration of camphor (0.25 mM) reduced the duration of afterhyperpolarization and disrupted the spontaneous rhythmic activity, which was evidenced by an increase in the coefficient of variation of interspike intervals. The medium concentration of camphor (0.5 mM) induced more disruption in the precision of spontaneous action potential and increased the frequency of firing along with a reduction of action potential falling slope and afterhyperpolarization. Neurons showed paroxysmal depolarization shift and burst firing after exposure to camphor at high concentration (1.5 mM). We found that the blockade of K^+ channels and upregulation of Ca^{2+} inward currents is essential for camphor-induced epileptiform activity, but the Na^+ currents and ion channel phosphorylation with protein kinases A and C are not required. This work provided novel evidence at cellular and subcellular level that the modulation of ion channels, especially direct inhibition of K^+ channels, is mechanistically involved to proconvulsive action of camphor.

1. Introduction

Herbal products are widely used around the world to improve general health or to treat certain diseases. Many of these products have potential toxicity when used inappropriately. Nevertheless, they are usually sold over-the-counter without any safety profile. The easy accessibility and the public assumption that the herbal remedies are safe have raised their popularity and also the incidence of undesired side effects and life-threatening intoxications. Furthermore, in most cases the active ingredients and the exact mechanisms underlying either beneficial or harmful effects of herbal remedies are not well-known (Grosset and Grosset, 2004).

Camphor ($C_{10}H_{16}O$) is a bicyclic monoterpenoid obtained from the wood of the *Cinnamomum camphora* L., native to East Asia. It has a long history of use as analgesic, antiseptic, antipruritic, stimulant, abortifacient and lactation suppressant. Today, camphor is an active ingredient in many ointments and inhalants that especially used to treat cold and musculoskeletal pain (Burkhart and Burkhart, 2003; Zuccarini, 2009). Topical application of camphor on the skin induces warmth sensation that is mediated by activation of the transient receptor potential vanilloid type 3 (TRPV3) channel (Green, 1990; Moqrich et al., 2005). Camphor also activates and subsequently desensitizes the TRPV1. Camphor-induced desensitization of TRPV1 has been suggested to contribute to its analgesic action (Xu et al., 2005).

Several reports describe serious toxicity or even death resulting from accidental ingestion or wrong administration of camphor/camphor-containing products (Agarwal and Malhotra, 2008; Mascie-Taylor et al., 1981; Patra et al., 2015). Camphor is rapidly absorbed from the gastrointestinal tract and the symptoms appear shortly after ingestion. Irritation of oral mucous membrane, abdominal pain, nausea, vomiting and ataxia are among the most common symptoms but the most prominent manifestation is convulsion. Death can result from respiratory failure or status epilepticus (Kopelman et al., 1979; Manoguerra et al., 2006).

* Corresponding author. Fax: +98 7132280916.
E-mail address: vatanparast@shirazu.ac.ir (J. Vatanparast).

http://dx.doi.org/10.1016/j.neuro.2015.12.012
0161-813X/

Cumulative doses of camphor used to be administrated to psychiatric patients to induce convulsion, which was later dropped in favor of electroconvulsive therapy (Pearce, 2008).

The membrane ion channels are the essential regulators of neuronal excitability that are targeted by many fast-acting convulsants. The rapid onset of camphor-induced convulsion suggests that the interaction of this monoterpenoid with ion channels may contribute to this action. The mechanism(s) that contribute to the epileptogenic action of camphor in central neurons has not been understood. Aside from the known action on TRP channels, a case study has suggested that camphor interacts with hyperpolarisation-activated channels in the axons of peripheral motor nerves and affects their electrophysiological properties after camphor toxicity (Jankelowitz et al., 2009).

Central snail neurons express a variety of ion channels that are responsible for normal electrical activity and also give them the ability to generate epileptiform discharges when treated with epileptogenic drugs like pentylenetetrazole and potassium channel blockers (Chen et al., 2000; Janahmadi et al., 2008; Onozuka et al., 1990). The ion channels usually show structural conservation in the ion selectivity filter elements, voltage sensor domains and the binding sites for natural ligand, pharmacological agents and toxins. The functional attributes show even more conservation across related ion channels in the living organisms and reflect the structural conservation (Baumann et al., 1988; Ruta et al., 2003). The manifestations of epileptiform activity, paroxysmal depolarization shift (PDS) and associated burst firing, show basic similarities in snail neurons and mammalian neurons, supporting the generally accepted idea that essential mechanisms underlying the epileptiform activity are constant in whatever nervous systems it appears. Furthermore, there are some technical advantages of using snail neurons when compared to mammalian preparations. Among others is the structural and functional intactness of the ganglia kept under *in vitro* conditions, while the slicing of mammalian brain is more damaging and disrupts the normal input and outputs. Because of their slow metabolic rate, the snail neurons also could be kept easier and for much longer periods in the laboratory condition and show less “run down” (Altrup et al., 1992, 2003; Chen et al., 2006; Onozuka et al., 1990).

In several studies, these neurons have been employed as model to explore the fundamental processes that control membrane excitability and may transform the normal activity into epileptiform activity when interrupted (Altrup et al., 2003; Janahmadi et al., 2008; Onozuka et al., 1991). We used conventional intracellular recording techniques to study the effect of camphor on the activity of snail neurons. The modulatory action of camphor on action potentials was analyzed according to the known role of different ion channels involved in the modulation of action potential waveform, rate of firing and pattern of activity.

2. Materials and methods

2.1. Animals and preparations

All experiments were performed on neurons in the subesophageal ganglia of land snail *Caucasotachea atrolabiata*. Animals were treated in accordance with the European Community guidelines and the experimental protocols were reviewed and approved by the animal care committee of the Shiraz University. 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 stripped off with fine forcipes.

2.2. Intracellular recording

SEC-10LX amplifier (npi electronic, Tamm, Germany) and Patchmaster 2 × 73.1 software (HEKA, Lambrecht, Germany) were used to record the membrane potentials and to inject current under current clamp conditions. Low resistance electrodes (1–5 MΩ) were pulled from filamented borosilicate glass (WPI, Sarasota, USA) using a P97 puller (Sutter Instruments, USA) and filled with 3 M KCl. Data were digitized using a LIH 8 + 8 data acquisition interface and analyzed by Fitmaster software (HEKA, Lambrecht, Germany). Neurons with stable resting membrane potentials (RMP) more negative than −38 mV were used for data collection. The RMP was defined as the mean voltage during interspike intervals, excluding the decaying phase of afterhyperpolarization (AHP) and the depolarizing ramp that precedes each action potential. Hyperpolarizing current steps (−1 to −5 nA at 1 nA increments, 500 ms pulse duration) were injected into the neurons and steady-state voltage changes from RMP were plotted against the injected currents. The input resistance (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 and its duration was measured at threshold level. The amplitude of AHP 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 regularity of interspike intervals (ISIs) of spontaneous action potentials in different conditions was assessed during 20 s recording periods using the coefficient of variation (CV = standard deviation of ISIs/mean ISI).

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. Camphor (96%), tetraethylammonium (TEA), 4-aminopyridine (4-AP), dimethyl sulfoxide (DMSO), nifedipine, N-[2-(p-Bromocinnamylamino) ethyl]-5-isoquinolinesulfonamide dihydrochloride (H-89) and chelerythrine were purchased from Sigma (St. Louis, MO, USA). Other chemicals obtained from Merck (Darmstadt, Germany). Camphor was prepared as 1 M stock in DMSO and diluted in snail Ringer before being added to the recording chamber. The final concentration of DMSO in the bathing solutions was always less than 0.2% and preliminary experiments showed these concentrations of DMSO do not alter the firing frequency and electrical properties of snail neurons. Stock solutions of H-89 and chelerythrine were made in DMSO 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. Stock solutions of ethosuximide and $NiCl_2$ were prepared in double distilled water. TEA-containing Ringer was prepared by replacing NaCl with equimolar amounts of TEA.

2.4. Statistical analysis

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