Physiology & Behavior 131 (2014) 93–98

Contents lists available at ScienceDirect

Physiology & Behavior

journal homepage: www.elsevier.com/locate/phb

Down regulation of sodium channels in the central nervous system of hibernating snails

T. Kiss *, I. Battonyai, Z. Pirger

Chemical-Ecology and Neurobiology Group, Department of Experimental Zoology, Balaton Limnological Institute, Centre for Ecological Research, Hungarian Academy of Sciences, Klebelsberg Kuno Str. 3., Tihany 8237, Hungary

HIGHLIGHTS

- We studied the regulation of voltage-gated sodium channel (Na_V) in hibernating snail.
- Expression of the $Na_V1.8$-like channels were down regulated during hibernation.
- The $Na_V1.9$-like channels were presented independently from various activity states.
- The mechanisms of the "channel arrest" could be different in diverse Na_V channel.

ARTICLE INFO

Article history:
Received 6 January 2014
Received in revised form 26 March 2014
Accepted 14 April 2014
Available online 24 April 2014

Keywords:
Adaptation
"Channel arrest"
Hibernation
Sodium channel down-regulation
Snail

ABSTRACT

Hibernation, as behavior, is an evolutionary mode of adaptation of animal species to unfavorable environmental conditions. It is generally characterized by suppressed metabolism, which also includes down regulation of the energy consuming ion-channel functioning. Experimental data regarding decreased ion-channel function are scarce. Therefore, our goal was to study the possible down regulation of voltage-gated sodium channel (Na_V) subtypes in the neurons of hibernating snails.

Our immunohistochemical experiments revealed that the expression of $Na_V1.8$-like channels in the central nervous system was substantially down regulated in hibernating animals. In contrast to $Na_V1.8$-like, the $Na_V1.9$-like channels were present in neurons independently from hibernating and non-hibernating states. Our western blot data supported the immunohistochemical results according to which the band of the $Na_V1.8$-like channel protein was less intensively labeled in the homogenate of the hibernating snails. The $Na_V1.9$-like immunoreactivity was equally present both in hibernating and active snails. Micro-electrophysiological experiments show that in hibernating snails both $Na_V1.8$- and $Na_V1.9$-like currents are substantially decreased compared to that of the active snails. The contradictory electrophysiological and immunohistochemical or western blot data suggest that the molecular mechanisms of the "channel arrest" could be different in diverse Na_V channel subtypes.

Climate changes will affect temperature extremes and a question is how different species beyond their physiological tolerance will or able to adapt to changing environment. Hibernation is an important mode of adaptation to extreme climatic variations, and pursuant to this the present results may contribute to the study of the behavioral ecology.

1. Introduction

In autumn and winter, when environmental factors such as the light, ambient temperature and food availability decrease, land snails go into an inactive dormant state, or hibernation. They look for a protected place among litter or burrow themselves in the ground to ensure their survival through the winter months. Snails withdraw into their shell, seal it with an operculum and stop feeding, which greatly reduces the oxygen consumption and the heart rate [1]. Generally, hibernating animals exhibit overall metabolic suppression, which is characteristic during the onset of and maintenance of the hibernating state. This decreased metabolic rate is the main strategy for invertebrate and vertebrate animals to overcome unfavorable environmental conditions such as anoxia, estivation and hibernation [1,2]. It is suggested that such an entropy-slowing strategy increases the survival potential of different species and developed during evolution [3]. Although the mechanisms of survival are known to include changes in the monoamine content, large stores of glycogen and drastically decreased metabolism [4,5], other mechanisms, such as the release of inhibitory transmitter, changes in peptide–protein profile and regulation of ion channel expression in excitable membranes have also been demonstrated or hypothesized

* Corresponding author. Tel.: +36 87 448 244/220.
E-mail address: kiss.tibor@okologia.mta.hu (T. Kiss).

http://dx.doi.org/10.1016/j.physbeh.2014.04.026
0031-9384/

[6–10]. It is observed that during hibernation, dendritic branching and synaptic profiles decrease in their complexity, size and numbers, but these rapidly re-emerge upon arousal [11,12]. Further on, hibernation induces a reduction of the firing activity of neurons, an increase of the intracellular Ca^{2+} concentration, a change in saccharide and amino acid concentration and an accumulation of various cryoprotectant substances [13–16]. For example, the cardiac action potential in hibernating chipmunks has no plateau as a result of down regulation of L-type Ca^{2+} channels, contrary to non-hibernating animals [17,18]. The hibernator phenotype also appears to have altered Na^{+} channel activity [19]. All the examples suggest that the ion "channel arrest," i.e., functional down-regulation of ion conducting channels, is important for surviving hypoxia and for cold tolerance in hibernating animals [20]. Electrophysiological studies providing in depth characterization of the "channel-arrest" are scarce. The only direct evidence for the modulation of voltage-gated sodium channels (Na_V) results from an experiment whereby a decline in the density of Na_V channels in the isolated turtle cerebellum was observed in anoxia [8]. The understanding of genetic and environmental underpinnings of hibernation is of increasing of concern in light of ongoing climate change. Therefore, the aim of this work was to test the hypotheses (1) that ion-channel density decreases during hibernation and (2) that down regulation of sodium channels may provide a basis for the electrical depression and energy conservation that contribute to the tolerance of hibernating snail brains.

2. Materials and methods

2.1. Electrophysiology

Micro-electrophysiological and immunohistochemical experiments were carried out on identified neurons of the central nervous system (CNS) of adult specimens of active and naturally hibernated land snail, *Helix pomatia*. One group of animals ($n = 30$) was kept active under wet conditions (relative humidity 95–98%) in a terrarium at room temperature (24 ± 2 °C) with a day/night cycle of 12:12 h and fed on lettuce or cucumber twice a week. The other group of snails collected in autumn, when the temperature falls under 8–10 °C, and stored outdoor in cages until they developed an epiphragm and became naturally hibernated. Thereafter, the hibernated animals were stored at 4 ± 2 °C (relative humidity 20–25%). Experiments on the neurons of hibernating and non-hibernating animals were performed at ambient temperatures between 20 and 22 °C. All procedures on snail was performed in accordance with the Hungarian Council on Animal Care guidelines on the ethical use of animals. Efforts were made to minimize both suffering and number of animals used in the experiments.

The CNS containing cerebral (CG), pedal (PG) and buccal ganglia (BG) was dissected from the animal, pinned in Sylgard-lined dish, and the thick connective tissue was removed. The perineurium was then softened with a 1% protease treatment (V/V; Sigma XIV, Sigma) for 5–8 min and exposed for penetration with glass microelectrodes.

Similar to previous work [21], current recordings of identified neurons expressing $Na_V1.8$- and $Na_V1.9$-like channels were performed using a GeneClamp amplifier (Axon Instruments, Union City, USA), designed to record currents from large diameter cells in two microelectrode voltage-clamp (VC) mode. Electrodes were pulled from filamented borosilicate glass capillaries (1B1150F-3 World Precision Instrument, Inc., Sarasota, USA) and had a resistance of 4–6 MΩ when filled with 2.5 M KCl solution. Data acquisition and analysis were performed using DigiData interface and pCLAMP software (Axon Instruments). Linear leak currents were subtracted from all data applying analogue compensation. To study Na_V-currents, while blocking K^{+} and Ca^{2+} currents, we used a modified physiological solution containing (in mM) NaCl 90, KCl 4, $CaCl_2$ 1, $MgCl_2$ 5, tetraethylammonium chloride (TEA-HCl) 30, 4-aminopyridine 4, Tris–HCl 10 and 50 μM $CdCl_2$ dissolved in distilled water (pH 7.4).

2.2. Western blot

The CNS was homogenized in sodium dodecyl sulfate (SDS) buffer, consisting of 1 M Tris–HCl, pH 6.8, 2% SDS, 10% glycerin and 1% mercaptoethanol in distilled water. Homogenized samples were cleared by centrifugation at 13000*g* for 10 min at 4 °C. Thereafter, the supernatant was resuspended in SDS sample buffer (50 mM Tris–HCl, pH 6.8, 2% SDS, 0.1% bromophenol blue, 10% glycerin and 100 mM dithiothreitol). The mixture was denaturized at 95 °C, and samples were loaded and protein extracts separated on 10% SDS–polyacrylamide gels (60 μg/lane) and then electrophoretically transferred to 0.45 μm PVDF membranes (Millipore). Membranes were blocked with 5% (w/v) non-fat dried skimmed milk in 12 mM Tris–HCl (pH 7.4), 160 mM NaCl and 0.1% Triton X-100 for 1 h. The membranes were then incubated with primary anti-$Na_V1.9$ (ASC-017, Alomone) or anti-$Na_V1.8$ (ASC-016, Alomone) at 1:200 dilutions overnight at 4 °C. Thereafter, the membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG secondary AB (DAKO, 1:2500). Blots were visualized in 0.05 M Tris–HCl (pH 7.6) containing 0.05% 3,3-diaminobenzidine (Sigma) and 0.01% H_2O_2 solution, after successive washing in cold PBS. Densitometric analysis was performed using Fiji image processing software. Values for the PACAP38-like bands were normalised relative to their corresponding β-actin bands used as internal standard. The analysis was repeated three times with brain samples obtained from separate batches of animals.

2.3. Immunohistochemistry (IHC)

For Na_V channel IHC, the CNS was dissected as described earlier (except protease treatment) and covered by cold fixative containing 4% paraformaldehyde diluted in 0.1 M phosphate-buffered saline (PBS, pH 7.4) overnight at 4 °C. Samples were then incubated for 4 h at 4 °C in PBS containing 25% sucrose. Thereafter, 14–16 μm thick cryostat sections were cut and placed on chrome-alum-gelatin coated slides. The cryosections were washed for 30 min in PBS containing 0.25% TX-100 at room temperature, and nonspecific binding sites were blocked by 0.25% bovine serum albumin (BSA) and 4% normal goat serum diluted in PBS-TX. Thereafter, sections were incubated with polyclonal rabbit anti-$Na_V1.9$ (ASC-017, Alomone) or anti-$Na_V1.8$ (ASC-016, Alomone) diluted in PBS-TX-BSA for 24 h at 4 °C. After washing three times in PBS-TX, the sections were incubated in secondary AB (FITC-conjugated polyclonal swine anti-rabbit IgG; Dako, Glostrup, Denmark; diluted 1:40 or donkey anti-rabbit IgG NorthernLights 493 fluorochrome-labeled antibody; R&D System) diluted 1:200 in PBS-TX-BSA for overnight at 4 °C. Finally, sections were washed in PBS and mounted in fluorescent mounting medium (Dako). The specificity of each of the ABs was tested by applying the method control and preadsorption test. In the case of the method control, BSA was used instead of primary or secondary AB. In the re-adsorption test, the diluted ABs were mixed with their blocking peptide as a control antigen (Alomone Laboratory, AG-01, 1 μg peptide/1 μg AB) and gently shaken overnight at 4 °C. Immunostaining was not observed either in the case of the method control experiments or the preadsorption control.

3. Results

Experiments made using the $Na_V1.8$ antibody revealed that most of the small diameter cells of the snail procerebrum (PC) possess $Na_V1.8$-like channels in addition to the fast Na_V, L-type Ca^{2+} and different K^{+} channels described recently [22]. An interesting observation was that the expression of the $Na_V1.8$-like channels in the PC and also throughout the CNS was substantially down regulated in the hibernating animals. Fig. 1 shows the distribution of the $Na_V1.8$ immunopositive elements in the PC of the non-hibernating (Fig. 1A) and hibernating (Fig. 1B) snails. It is clearly demonstrated that $Na_V1.8$-like immunolabeled cells are almost absent in hibernating and are abundant in non-hibernating