Neuroscience 203 (2012) 78–90

CELLULAR LOCALIZATION AND KINETIC PROPERTIES OF $Na_V1.9$-, $Na_V1.8$-, AND $Na_V1.7$-LIKE CHANNEL SUBTYPES IN *HELIX POMATIA*

T. KISS,[a]* Z. LÁSZLÓ[a] AND Z. PIRGER[a,b]

[a]*Department of Zoology, Balaton Limnological Research Institute, Hungarian Academy of Sciences, Tihany, Hungary*

[b]*School of Life Science, University of Sussex, Falmer, Brighton, UK*

Abstract—This article concerns the kinetics, selectivity, and distribution of the $Na_V1.9$, $Na_V1.8$, and $Na_V1.7$ channel subtypes in the CNS of the snail, *Helix pomatia*. Within the snail brain, $Na_V1.9$- and $Na_V1.8$-like channel subtypes are widely expressed, with particularly high levels in the pedal, cerebral, and buccal ganglia. The suboesophageal ganglion contains equal amounts of neurons labeled with $Na_V1.9$, 1.8, and 1.7 antibodies. Our data show that different types of ion channels are localized to discrete neurons and regions of the neuronal membrane affecting by this way the physiology of synaptic transmission or nerve conduction. Based on the voltage dependence and kinetics, the non- or slowly inactivating currents were observed in identified and nonidentified neurons of the snail CNS attributed to separate Na-channel subtypes. These observations provide the first evidence for the presence of the composite Na-current in snail neurons. The significance of $Na_V1.9$ channels in gastropod neurons is assigned to regulating the subthreshold membrane depolarization. First time, we have demonstrated that in addition to the $Na_V1.2$-like channels most of the neurons contain $Na_V1.8$- or 1.7-like channels carrying the composite inward sodium current. In this way, neurons containing different sets of channels differently are regulated, which allows further dynamic modulation of neuronal activity. The neuronal soma membrane revealed low ion selectivity of the Na-channels with slow kinetics, which is a general property of gastropod molluscs. In addition, the relative similarity of the biophysical properties of voltage-gated currents between vertebrates and invertebrates may reflect a structural similarity existing between Na-channel subtypes pointing to a common evolutionary origin.

Key words: mollusk, neuron, Na-channel subtypes, Na-channel expression, biophysical properties.

Voltage-gated Na-channels (VGNC) play an outstanding role in the excitability of neurons because they allow initiation and propagation of action potentials. VGNCs are complex, heterotrimeric proteins consisting of the highly conserved large central pore forming α-subunit and one or two smaller auxiliary β-subunits (Catterall, 1993). The α-subunit is encoded by 10 distinct genes, giving rise to a Na_V 1.1–1.9 and Na_X channel subtypes in vertebrates. Many of these channels seem to have a specific developmental and tissue distribution or cellular localization. Different isoforms of Na-channel α-subunit perform distinct function as has been inferred from correlating electrophysiological and histochemical data. For example, in Purkinje cells three isoforms are present the $Na_V1.1$, and 1.2 carrying transient and $Na_V1.6$ carrying persistent component of the Na-current (Raman and Bean, 1999). The rat dorsal root ganglion (DRG) neurons contain at least six Na-channel subtypes, including $Na_V1.1$, 1.6, 1.7, 1.8, 1.9, and Na_X (Caffrey et al., 1992; Roy and Narahashi, 1992; Renganathan et al., 2002; Coste et al., 2004). Therefore, the inward Na-current in most of the vertebrate neurons seems to be a mixture of several VGNC subtypes. In invertebrates, the biophysical properties, pharmacology, and gene organization of Na-channels are largely similar to the vertebrates, supporting the view that the ancestral Na-channel has been established long before the evolutionary separation of the invertebrates and the vertebrates (Goldin, 2002).

The degree of similarity between vertebrate $Na_V1.9$ and different invertebrate Na-channel α-subunits shows 60–70% sequence homology, suggesting that the sequence and overall domain organization are largely the same (Liu et al., 2001; Kiss, 2008). The sequencing data concerning the invertebrate Na-channels, including molluscs, however are much more incomplete compared with mammalian Na-channels. Earlier, it was reported that the Na-current (I_{Na}) in a randomly selected small diameter gastropod neurons has slower activation kinetics than that in the vertebrates or even in the other invertebrates. The slow kinetics of gastropod Na-channel are evident however over limited voltage range. Slow Na-channels are also present in giant neurons of terrestrial, aquatic, and marine gastropods, where they represent functional adaptations evolved during evolution (Gilly et al., 1997). In all cases, the I_{Na} activates and inactivates more slowly than that in the giant axon of squid, a cephalopod mollusc (Kostyuk et al., 1977; Meves, 1978; Adams and Gage, 1979; Adams, 1980; Gilly et al., 1997). Recently, the persistent Na-current (I_{NaP}) was also observed in the neurons of gastropods (Staras et al., 2002; Kiss, 2003) and in giant axon of squid (Clay, 2003), and it was suggested that channel carrying the I_{NaP} could be a separated channel subtype (Nikitin et al., 2008) although it was not demonstrated by immunohistochemistry. The I_{NaP} plays an important role in boosting the excitatory synaptic input, setting the resting membrane potential (MP) and the threshold for spiking regulating thereby the integrative or pacemaking properties of neurons (Llinás, 1988; Herzog et al., 2001; Kiss, 2008). An

*Corresponding author. Tel: +3687448244.
E-mail address: kisst@tres.blki.hu (T. Kiss).

Abbreviations: AB, antibody; BSA, bovine serum albumin; DRG, dorsal root ganglion; ELISA, enzyme-linked immunosorbent assay; IHC, immunohistochemical; PBS, phosphate-buffered saline; TMA, tetramethylammonium; TEA, tetraethylammoniumchloride; VGNC, voltage-gated Na-channels.

0306-4522/12 $36.00 © 2011 IBRO. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.neuroscience.2011.11.045

Table 1. Relation of the used antisera indicating the acronyms, epitope, reference number, and dilution in our experiment

Antibody types	Acronyms	Epitope	Alomone prod. #	Used dilution
Anti-$Na_V1.9$	*SNS2*	1748–1765 of rat $Na_V1.9$	ASC-017	1:200
Anti-$Na_V1.8$	*SCN10A*	1943–1957 of rat $Na_V1.8$	ASC-016	1:200
Anti-$Na_V1.7$	*SCN9A*	446–460 of rat $Na_V1.7$	ASC-008	1:200
Anti-$Na_V\beta2$	*SCN2B*	197–215 of rat $Na_V\beta2$	ASC-007	1:200
Anti-PanNa_V	*SP19*	1500–1518 of rat $Na_V1.1$	ASC-003	1:200

increased expression of the I_{NaP} was shown in response to classical conditioning both in *Lymnaea* and *Helix* suggesting the role of I_{NaP} in the associative learning and memory formation (Nikitin et al., 2006; Nikitin et al., 2008; Kiss et al., 2009). To date, there is a lack of data defining the distribution of different VGSC subtypes in molluscs including gastropods. This is in part due to the deficient sequence information for the gastropod Na-channel orthologous and the lack of availability of antibodies (ABs) developed against Na-channels of molluscs. In contrast, the distribution studies are important for basic understanding of Na-channel function both from comparative and evolutionary aspects. It is now evident that each of the Na-channel subtype has its own unique cell type- and subcellular compartment-specific distribution on the surface of nerve cells. Knowledge of such distributions would provide further information on the diversity of neuronal types and how they are important in understanding the dynamic modulation of neuronal networks.

In the present study, we have systematically examined and compared the biophysical properties and distribution of $Na_V1.9$-, 1.8-, and 1.7-like isoforms in the nervous system of the terrestrial gastropod, *Helix pomatia*. The data will extend our knowledge in interpretation of neuronal activity of different neurons expressing different Na-channel subtypes and in understanding the function of neuronal networks involved in different behaviors.

MATERIALS AND EXPERIMENTAL PROCEDURE

Preparation

Electrophysiological and immunohistochemical (IHC) experiments were carried out on the cells of the CNS of adult specimens of *Helix pomatia*. The CNS containing suboesophageal, cerebral, and buccal ganglia was dissected from the animal, pinned out in Sylgard-lined dish, and the thick connective tissue was removed. Then, the preparation was treated with 1 mg/ml protease type XIV (Sigma-Aldrich, Budapest, Hungary), dissolved in normal physiological saline (in mM: NaCl 80, KCL 4, $CaCl_2$ 10, $MgCl_2$ 5, and Tris-HCl 10, adjusted to pH 7.4 with NaOH), for 5–8 min at room temperature (~22 °C). The protease-treated preparation was washed thoroughly and kept at 4 °C for 20–30 min. Thereafter, the thin connective tissue layer surrounding the ganglia was removed by fine forceps, enabling subsequent impalement with microelectrodes. The preparation was continuously perfused with normal physiological solution at a rate of approximately 1 ml/min, allowing the complete exchange of the recording chamber in 2 min.

IHC detection of Na-channels

For Na-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) for overnight at 4 °C. Samples were then incubated for 4 h at 4 °C in PBS containing 25% sucrose and thereafter 14–16-μm thick cryostat sections were cut and placed on chrome alum gelatin-coated slides. The cryostat sections 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 ABs (Table 1) 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 both 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 applying the method control and preadsorption test. In case of method control, BSA was used instead of primary or secondary AB. In preadsorption 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 case of method control experiments or preadsorption control.

Electrophysiology

Electrophysiological recordings of identified neurons expressing $Na_V1.7$, 1.8, or 1.9 channels were performed using a GeneClamp amplifier (Axon Instruments, Union City, USA), designed for recording 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) with vertical puller (David Kopf, Model 730, Tujunga, CA, USA) and had a resistance of 4–6 MΩ when filled with Na-acetate or 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. For studying Na-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, tetraethylammoniumchloride (TEA-HCl) 30, 4-aminopyridine 4, Tris-HCl 10, and 50 μm $CdCl_2$ dissolved in distilled water (pH 7.4). For the activation—conductance plots, the Na^+ conductance (g_{Na}) was calculated from the peak current (I_{Na}) according to the relation $g_{Na}=I_{Na}/(V\text{-}E_{Na})$, where V is the test potential and E_{Na} the reversal potential. The activation—conductance plot was fitted using the Boltzmann equation: $g/g_{max}=1/[1+\exp(V_{½}–V)/k]$, where g is the normalized conductance relative to the maximum conductance (g_{max}), $V_{½}$ is the MP at which half the channels are activated and k is the slope of the curve. In the inactivation curves, the currents were normalized (I) to the maximum ($I_{max.}$) current in each experiment and plotted against the conditioning potentials. The inactivation curves were fitted by the Boltzmann equation: $I/I_{max.}=1/[1+\exp(V_{½}–V)/k]$, where V is the conditioning pulse potential, $V_{½}$ is the MP at which half the channels are inactivated and k is the slope. The recovery from inactivation is approximately described by an exponential function