NeuroToxicology 60 (2017) 142–149

Contents lists available at ScienceDirect

NeuroToxicology

Full length article

Functional reconstitution of rat $Na_v1.6$ sodium channels *in vitro* for studies of pyrethroid action☆

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ARTICLE INFO

Article history:
Received 7 January 2016
Received in revised form 17 March 2016
Accepted 17 March 2016
Available online 21 March 2016

Keywords:
*Xenopus oocytes*m
HEK293 cells
Voltage clamp
Tefluthrin
Deltamethrin
S-bioallethrin

ABSTRACT

The ability to reconstitute sodium channel function and pharmacology *in vitro* using cloned subunits of known structure has greatly enhanced our understanding of the action of pyrethroid insecticides at this target and the structural determinants of resistance and interspecies selectivity. However, the use of reconstituted channels raises three critical questions: (1) Which subunits and subunit combinations should be used? (2) Which heterologous expression system is preferred? (3) Which combination of subunits and expression system best represents the function of native neuronal channels in the organism of interest? This review considers these questions from the perspective of recent research in this laboratory on the action of pyrethroid insecticides on rat $Na_v1.6$ sodium channels by comparing the effects of heteroligomeric complex composition on channel function and insecticide response when channels are expressed in either *Xenopus* oocytes or stably-transformed HEK293 cells. These comparisons provide new insight into the influence of cellular context on the functional and pharmacological properties of expressed channels, the modulatory effects of sodium channel auxiliary subunits on the action of pyrethroids, and the relative fidelity of the *Xenopus* oocyte and HEK293 cell expression systems as model systems for studying of channel function and pyrethroid action.

1. Introduction

Pyrethroids owe their insecticidal activity to their ability to modify the gating of voltage-gated sodium channels (VGSCs), which mediate the transient increase in the sodium permeability of the nerve membrane that underlies the rising phase of the nerve action potential (Bloomquist, 1993; Soderlund, 1995; Narahashi, 1996). The identification of single amino acid substitutions in the VGSC sequences of resistant insects that reduce the susceptibility of expressed channels to pyrethroid modification provides further evidence that action on VGSCs underlies the primary insecticidal actions of pyrethroids (Soderlund and Knipple, 2003; Soderlund, 2005; Rinkevich et al., 2013).

The compelling evidence for effects on VGSCs as the mechanism of insecticidal activity of pyrethroids and the strong conservation of VGSC structure, function and pharmacology across animal taxa (Goldin, 2002) implicates VGSCs in the central nervous system (CNS) as important target sites for the acute neurotoxic effects of pyrethroids in mammals. However, individual CNS neurons express multiple VGSC isoforms and contain multiple functionally and pharmacologically distinct VGSC heteromultimeric complexes (Felts et al., 1997; Whitaker et al., 2000, 2001). Thus, the relative sensitivity of different isoforms and subunit complexes to pyrethroids, and therefore the relative importance of these isoforms and complexes as targets in intoxication, cannot be determined in studies using native neurons. This difficulty can be overcome by using *in vitro* systems for the heterologous expression and functional characterization of VGSC complexes of defined subunit structure.

This brief review summarizes and synthesizes work from this laboratory during the past decade using two heterologous expression systems – the unfertilized oocytes of the frog *Xenopus laevis* and the human embryonic kidney-derived HEK293 cell line – to express the rat $Na_v1.6$ sodium channel, either alone or in combination with the rat β1 and β2 auxiliary subunits, and characterize both their functional properties and their pharmacological modification by pyrethroid insecticides. We also provide a

☆ This paper is based on a contribution to the symposium "Insecticide Action on Ion Channels: A Tribute to Toshio Narahashi" in the Division of Agrochemicals at the 250th American Chemical Society National Meeting on August 16, 2015.

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http://dx.doi.org/10.1016/j.neuro.2016.03.010
0161-813X/

provisional assessment of the relative merits of these two systems for predicting the action of pyrethroids on VGSCs in their native neuronal environment.

2. Structural and pharmacological heterogeneity of mammalian sodium channels

2.1. Structural heterogeneity

Potential VGSC targets for pyrethroid intoxication in mammals comprise nine different pore-forming α subunit isoforms ($Na_v1.1$–$Na_v1.9$) that exhibit unique patterns of developmental and anatomical expression and varied functional and pharmacological properties (Goldin, 2001). Four α subunits ($Na_v1.1$, $Na_v1.2$, $Na_v1.3$, and $Na_v1.6$) are abundantly expressed in either the embryonic or adult brain and represent possible targets for pyrethroid neurotoxicity in the CNS. The $Na_v1.3$ and $Na_v1.6$ isoforms are of particular interest because they are the most abundantly-expressed isoforms in the embryonic ($Na_v1.3$) and adult ($Na_v1.6$) brain (Felts et al., 1997; Whitaker et al., 2000, 2001; Shah et al., 2001).

Additional diversity among sodium channels results from the coassembly in the nerve membrane of an α subunit with one or two auxiliary β subunits that modulate channel gating and regulate expression (Isom, 2001). VGSCs in the adult brain are heterotrimeric complexes of one α subunit and two β subunits that differ in structure and their mode of association (covalent or noncovalent) with the α subunit (Hartshorne and Catterall, 1984). Although there are four β subunits in mammals, the ubiquitous expression of the β1 and β2 subunits in the adult brain implies that the majority of brain VGSCs are α+β1+β2 complexes (Whitaker et al., 2000; Shah et al., 2001; Schaller and Caldwell, 2003). However, the actual subunit composition of native sodium channel complexes remains to be determined.

2.2. Pharmacological heterogeneity

The correlation of pyrethroid sensitivity with mammalian VGSC structure using neuronal tissue preparations is complicated by the fact that neurons are now known to express multiple VGSC isoforms. However, a limited number of physiological studies suggest that sodium channel isoforms expressed in various mammalian tissues exhibit differential sensitivity to pyrethroids. The clearest evidence of the pharmacological heterogeneity among VGSC isoforms is found in the responses of the tetrodotoxin (TTX)-sensitive and TTX-resistant VGSC populations in dorsal root ganglion neurons to pyrethroids. The TTX-resistant sodium current in these cells is much more sensitive than the TTX-sensitive current to allethrin (Ginsburg and Narahashi, 1993), tetramethrin (Tatebayashi and Narahashi, 1994; Song et al., 1996) and deltamethrin (Tabarean and Narahashi, 1998).

Several studies have employed transient expression in *Xenopus laevis* oocytes to assess the action of pyrethroids on individual rat sodium channel isoforms and defined subunit complexes (Smith and Soderlund, 1998, 2001; Vais et al., 2000; Soderlund and Lee, 2001; Choi and Soderlund, 2006; Meacham et al., 2008; Tan and Soderlund, 2009, 2010, 2011a). Among the five rat isoforms examined to date, the $Na_v1.3$, $Na_v1.6$ and $Na_v1.8$ isoforms are relatively sensitive to pyrethroid modification; in particular, the $Na_v1.8$ isoform is likely responsible for the TTX-resistant, pyrethroid-sensitive current in dorsal root ganglion sensory neurons. By contrast the $Na_v1.2$ and $Na_v1.7$ isoforms are relatively resistant to pyrethroid modification.

The identification of $Na_v1.6$ as a pyrethroid-sensitive isoform is of particular interest because $Na_v1.6$ is the most abundant sodium channel α subunit in the adult rat brain (Auld et al., 1988), where it is preferentially expressed in regions of brain axons associated with action potential initiation (Hu et al., 2009). $Na_v1.6$ is also the predominant isoform at nodes of Ranvier and is expressed in presynaptic and postsynaptic membranes of the neocortex and cerebellum (Caldwell et al., 2000). Thus, $Na_v1.6$ sodium channels play important roles in both electrical and chemical signaling in the brain. The remainder of this review focuses on our studies of the function and pyrethroid pharmacology of the rat $Na_v1.6$ isoform, either alone or in complexes with the β1 and β2 auxiliary subunits, expressed either in *Xenopus* oocyte or HEK293 cells.

3. Rat $Na_v1.6$ sodium channels expressed in *Xenopus* oocytes

3.1. The Xenopus oocyte expression system

The *Xenopus* oocyte expression system is arguably the most widely-employed heterologous expression system for the reconstitution and study of both ligand-gated and voltage-gated ion channels (Goldin, 2006). When injected with synthetic mRNA the oocyte efficiently translates the message, performs post-translational modifications on the nascent protein, and inserts the mature protein into the cell membrane. For some channels, the oocyte system is the only heterologous expression system that will permit functional and pharmacological characterization *in vitro*. For example, all of our knowledge of heterologously-expressed insect VGSCs, including numerous studies identifying the functional role of putative insecticide resistance mutations (Rinkevich et al., 2013), is derived from the oocyte system, and all efforts to achieve the functional expression of insect VGSCs in other systems have so far failed.

The oocyte expression system is particularly well-suited to studies in which channel structure is the principal experimental variable. Expression in oocytes, when coupled with site-directed mutagenesis, also facilitates the testing of specific hypotheses regarding the effects of channel structure on both functional and pharmacological properties and the role of specific domains and amino acid residues in drug and insecticide binding to the channel.

Despite its considerable strengths the oocyte system also possesses two limitations that are intrinsic to the biology of the oocyte itself (Goldin, 2006). First, the biochemistry of post-translational modification is specific to the amphibian oocyte and therefore may differ markedly from the processing of proteins that normally reside in the membranes of mammalian or insect neurons. Second, the large cell surface area and yolk of the oocyte provide a significant sink for lipophilic compounds such as pyrethroids. As a result, oocytes continue to accumulate pyrethroid during perfusion for up to 3 h (Harrill et al., 2005); thus, nominal pyrethroid concentrations in the perfusion medium may not reach equilibrium during an experiment and also may not reflect the concentrations available to bind to expressed sodium channels. However, we found that the extent sodium channel modification by pyrethroids reached an apparent equilibrium after perfusion for 20 min (Choi and Soderlund, 2006), suggesting that a portion of the oocyte burden of pyrethroid is not accessible to channels that are expressed in the cell membrane. Nevertheless, the experimental benefits afforded by the oocyte system must be balanced against the uncertain extent to which the results obtained accurately reflect the properties of the same channels in their native neuronal environment.

3.2. Action of pyrethroids on rat $Na_v1.6$ sodium channels

We expressed the rat $Na_v1.6$ α subunit with the rat β1 and β2 subunits to give a channel complex in oocytes that reflected the inferred composition of the most abundant CNS complex and assessed the action of *S*-bioallethrin, tefluthrin and deltamethrin