Toxins That Affect Voltage-Gated Sodium Channels

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Contents

Abstract

Voltage-gated sodium channels (VGSCs) are critical in generation and conduction of electrical signals in multiple excitable tissues. Natural toxins, produced by animal, plant, and microorganisms, target VGSCs through diverse strategies developed over millions of years of evolutions. Studying of the diverse interaction between VGSC and VGSC-targeting toxins has been contributing to the increasing understanding of molecular structure and function, pharmacology, and drug development potential of VGSCs. This chapter aims to summarize some of the current views on the VGSC-toxin interaction based on the established receptor sites of VGSC for natural toxins.

Keywords

Binding sites · Toxins · Voltage-gated sodium channel

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1 Introduction

Voltage-gated sodium channels (VGSCs) initiate and conduct action potentials in neurons, cardiac and skeletal muscle cells, and some endocrine cells (Hodgkin and Huxley 1952; Catterall 1992; Zimmer et al. 2014; Rogart 1981; Wada et al. 2008; Donatsch et al. 1977). In general, VGSCs are composed of a pore-forming α subunit and accessary subunits (Catterall et al. 2005; Marban et al. 1998; Scheuer 1994; Wood and Baker 2001). The α subunit of VGSCs is consisted of four functional domains (I–IV). Each domain contains six transmembrane segments (S1–S6) in which S1–4 forms a voltage sensor component and S5–6 forms a pore component. In mammals, nine subtypes of functional α subunits of VGSC (Nav1.1–1.9) are expressed differentially at tissue, cellular, and subcellular levels (Catterall et al. 2005; Noda et al. 1986; Goldin 1999; Bao 2015). VGSCs play an important role in a variety of inherited and acquired diseases and have been serving as promising drug discovery targets in recent years (Cox et al. 2006; Dib-Hajj et al. 2010; Emery et al. 2016; Faber et al. 2012; Fertleman et al. 2006; Hong et al. 2004; Kohling 2002; Lai et al. 2003; George 2005). However, despite of enormous efforts spent to develop novel therapy drugs of VGSC blockers, success has been rare (Carnevale and Klein 2017). One reason behind this problem is the lack of in-depth and complete understanding of how VGSCs work and how ligands interact with VGSC.

Natural toxins targeting VGSCs are developed by animal, plant, and micro organisms over millions of years of evolution (Moran et al. 2009; Zakon 2012). These toxins are developed to capture the prey and/or to defend the predators through diverse interaction with VGSCs (Cestele and Catterall 2000; Wang and Wang 2003). So far, six receptor sites of VGSCs have been defined based on the interaction between VGSCs and natural toxins (Li and Tomaselli 2004; Nicholson 2007; Pedraza Escalona and Possani 2013; Hille 1968). Accordingly, six types of VGSC-targeting toxins have been grouped as follows: (1) site 1 toxins block VGSCs through the outer pore; (2) site 2 toxins modulate the gating, permeability, and selectivity of VGSCs involving the inner pore; (3) site 3 toxins inhibit the fast inactivation of VGSCs with subtype selectivity; (4) site 4 toxins facilitate the steady-state activation and reduce the amplitude of the peak currents of VGSCs with species selectivity; (5) site 5 toxins enhance the activation and inhibit the fast inactivation of VGSCs; and (6) site 6 toxins inhibit the fast inactivation of VGSCs through a binding site different from site 3. These diverse VGSC-targeting toxins have been and will continue to be unique tools for studying the structure and function of VGSCs and for developing VGSC-targeting strategies in VGSC-related diseases.

2 Toxins Binding to Site 1 of VGSCs

Among all putative binding sites on sodium channels, site 1 is probably the best defined and the most straightforward site. The site 1 is composed of residues on the reentrant P-loops from all four domains. It is occupied by two different groups of neurotoxins: the water-soluble heterocyclic guanidines tetrodotoxin (TTX) and saxitoxin (STX), and the peptidic toxins μ-contoxins (Catterall et al. 2007). TTX is

purified from the roe, ovaries, skin, and liver of at least 40 species of puffer fish (Fuhrman 1967). Moreover, it is also extracted from mollusks, crabs, octopus, fish, salamander, and Central American frogs (Sheumack et al. 1978; Noguchi et al. 1986; Daly et al. 1994; Mattei and Legros 2014). STX is produced by the marine dinoflagellate *Gonyaulax catenella*, and it is found in bivalves such as clams and mussels that feed on the dinoflagellates (Schantz 1986). μ-conotoxins are isolated from the venom of *Conus geographus* and related cone snails (Cruz et al. 1985; Sato et al. 1983). The binding of μ-conotoxins on receptor site 1 (Narahashi et al. 1964; Hille 1975) seems distinct from TTX. Several mutations in Nav1.4 affect the binding of TTX but not μ-conotoxins suggesting that the binding sites of TTX and μ-conotoxins are not identical (Stephan et al. 1994). Localization of TTX/STX as well as μ-conotoxins receptor sites has been instrumental in identifying the pore loop and clarifying the regions of VGSCs involved in the ion selectivity filter (Catterall et al. 2007).

TTX has been used for many decades to characterize the physiological function of sodium channels (Sato et al. 2001). According to the sensitivity to TTX, the nine VGSC subtypes can be classified into two groups, with blocking concentrations being in the nanomolar range for the TTX-S channels (Nav1.1, Nav1.2, Nav1.3, Nav1.4, Nav1.6, and Nav1.7) and in the micromolar range for the TTX-R channels (Nav1.5, Nav1.8, and Nav1.9). The structural determinant of TTX resistance has been located as a single amino acid residue found directly adjacent to the inner-ring residue in domain I. In TTX-S channels this residue is a tyrosine or phenylalanine, but in TTX-R channels it is a cysteine or serine (Goldin 2002). The neuronal TTX-R subtypes (Nav1.8 and Nav1.9) are preferentially expressed in the peripheral sensory neurons of DRG, trigeminal, and nodose ganglion. In DRG, Nav1.8 and Nav1.9 are preferentially expressed in nociceptive neurons. The activity of neuronal TTX-R channels can be studied in nociceptive neurons by application of sub-micromolar concentrations of TTX that selectively blocks TTX-S channels (Cummins et al. 2007). In addition to experimental tools, lower concentrations of TTX also show medical potentials as an anesthetic agent in treating pain, migraine, and withdrawal symptoms in heroin addicts (Narahashi 2008).

μ-Conotoxins contain several positively charged residues, which are important in interacting with the negatively charged residues of the selectivity filter. In particular, a conserved arginine at position 13 or 14 provides a guanidinium group, which appears to be the most critical for channel block (Keizer et al. 2003). On the one hand, because μ-conotoxins are much larger than TTX and STX while they bind at site 1, they are also possible to interact with other positions in VGSCs. On the other hand, residues that are important for TTX resistance in VGSCs appear not to be involved in the action of the μ-conotoxins, which suggested that the binding sites of TTX as well as μ-conotoxins are partially overlapped. Compared to TTX and STX, μ-conotoxins show subtype selectivity toward VGSCs. A group of μ-conotoxins composed by GIIIA, GIIIB, and GIIIC are highly effective blockers of the skeletal muscle sodium channel Nav1.4. However, several other μ-conotoxins, including SmIIIA, SIIIA, and KIIIA, are selective inhibitors of TTX-R sodium currents (Ekberg et al. 2008). For example, the SIIIA is able to block TTX-R sodium currents in rodent DRG neurons with little effect on TTX-S currents (Wang et al. 2006). The isoform specificity of μ-conotoxins seems

to be related to specific residues in the segments 5 and 6 in domain II of the VGSC. The subtype selectivity of conotoxins might potentially enable them as valuable tools for studying the physiological roles of sodium channels. Systemic administration of KIIIA demonstrated analgesic effects in the inflammatory pain model induced by formalin, mainly through inhibition of TTX-R sodium currents (Zhang et al. 2007).

Another neurotoxin believed to affect site 1 is *Phoneutria nigriventer* toxin 1 (Tx1) from *Phoneutria nigriventer*. Tx1 blocks sodium currents in a state-dependent manner and competes for binding with μ-conotoxins GIIIB but not with TTX (Martin-Moutot et al. 2006). Therefore it was suggested that Tx1, like TTX, binds to a micro site and that the binding site of μ-conotoxins overlaps the micro sites of TTX and Tx1 (Li et al. 2003).

3 Toxins Binding to Site 2 of VGSCs

Lipid-soluble neurotoxins that target this receptor site have diverse chemical structures and are not structurally related (Catterall 1980). Their structural non-relatedness is reflected in their different sources as they can come from plants, animals, and bacteria (Wang and Wang 2003). Site 2 neurotoxins from plants are alkaloids like veratridine (VTD; from *Liliaceae*) and aconitine (from *Aconitum napellus*) and grayanotoxins (GTX; from *Ericaceae*). Batrachotoxin (BTX) is abundant in the skin of Colombian frog *Phyllobates aurotaenia*. In addition, antillatoxin (Cao et al. 2008) and hoiamide (Pereira et al. 2009) that are isolated from some cyanobacteria (*Lyngbya majuscula*) were also found to target the neurotoxin receptor site 2. These toxins are useful tools in the study of voltage-gated Na^+ channels. For example, radioactive probes using these toxins, which exhibit high affinities toward Na^+ channels with relatively low nonspecific binding characteristics, had long been used to characterize the voltage-gated sodium channels directly or the allosteric modulation by different classes of neurotoxins in various excitable membranes.

Site 2 toxins are considered to be activators as their actions on sodium channels are in such a way that sodium channels open more easily and stay open longer. When Na^+ flux assays are used, BTX is considered as a full activator, whereas veratridine and aconitine are considered as partial activators. Site 2 toxins bind preferentially to the open state of sodium channels and alter the activity of sodium channels. Upon binding, the voltage dependence of activation is shifted toward more negative potentials, causing sodium channels to open at resting potentials. The inactivation is slowed down or inhibited resulting in sustained, non-inactivating currents via an allosteric mechanism. However, the sodium conductance is reduced by site 2 binding toxins. For instance, single-channel conductance of BTX-activated Na^+ channels and veratridine-activated Na^+ channels are about 50% and 25%, respectively, of that of normal Na^+ channel. The underlying mechanism for the reduction in single-channel conductance by these two neurotoxins is not known. One possibility is a partial block of the Na^+ channel permeation pathway by these two ligands. Additionally, the ion

selectivity of site 2 toxin-modified channels is altered possible due to a decreased discrimination for permeating ions (Tikhonov and Zhorov 2005; Du et al. 2011).

The mapping of the neurotoxin site 2 has shed light on the molecular determinants responsible for the alterations of channel gating upon toxin binding. By using photo-labeled BTX, it was identified that the first residues within the inner helixes of DI S6 (I433, N434, and L437) and DIV S6 (F1579 and N1584) are crucial for BTX binding in Nav1.4 (Wang and Wang 1998, 1999) and that DIVS6 (I1760 and F1764) (equivalent to F1579) are crucial for BTX binding in Nav1.2 (Linford et al. 1998). By using point mutations of the equivalent residues in Nav1.5, the key role of these residues in BTX binding was also confirmed (Wang et al. 2007). Although site 2 is generally believed to be localized mainly at the S6 of DI and DIV, it is now considered that specific amino acid residues among all four S6 segments are involved in the neurotoxin receptor site 2 by using site-directed mutagenesis (Wang et al. 2001). The exact location of all known molecular determinants contributing to the BTX binding site has been well reviewed elsewhere (Du et al. 2011). An initial allosteric model was proposed to explain the observed channel gating alterations upon site 2 toxin binding, in which the lipid-soluble toxins bind to lipid-exposed sites distinct from the pore and/or the voltage sensors (Catterall et al. 2007). Although this allosteric model provided a reasonable interpretation of modifications in channel gating, conductance, and ion selectivity, increasing evidence have emerged to promote the revision of this model. It was suggested that these site 2 toxins bind within the pore rather than at the lipid-exposed channel interface (Tikhonov and Zhorov 2005; Du et al. 2011). In a new revised model, site 2 neurotoxins, such as BTX, bind within the inner pore with residues in the S6 segments of all four domains, exposing the activator directly to the permeation pathway, which is also consistent with most studies on BTX binding (Du et al. 2011). This revised model also included the previous hypothesis that the observed inhibition of inactivation might be due to BTX interaction with the above-described residues in S6 of DIV (Du et al. 2011). The DIV S6 segment is not only involved in fast inactivation, while the altered movements of toxin-bound S6 segments also influence the movement of adjacent segments. Therefore, it is postulated that BTX binding alters the voltage-dependent movement of the DIV S4 voltage sensor and thereby modifies channel activation and its coupling to inactivation (Catterall et al. 2007). In addition, the reduced sodium conductance can be attributed to the outcome of a narrower Na^+ binding site due to the presence of a BTX in the inner pore. In contrast, the altered ion selectivity may be a direct consequence of a wider selectivity filter in the toxin-modified channels. It was established that the DEKA locus within the selectivity filter determines the sodium channel ion selectivity. Moreover, K1422 can be seen as a key residue because single point mutation of this residue into a glutamic acid conferred calcium conducting characteristics onto VGSCs (Heinemann et al. 1992). This new model suggests that BTX does not interact directly with the DEKA locus but rather causes a deficiency of water molecules in the proximity of the selectivity filter. The displacement of water molecules may lead to a shift in the PKA of the ion selectivity-determining residue K1422 and, in this way, lower the discrimination in permeating ions (Du et al. 2011).

For the lipid-soluble grayanotoxins (GTXs), several studies have contributed in locating their binding site. Similar to the BTX binding residues, it was found that S251, I433, N434, L437, I1575, and F1579 on Nav1.4 and their equivalents on Nav1.5 are involved in the binding of GTXs (Kimura et al. 2001; Ishii et al. 1999). Mutation of one specific residue at position 1586 in DIV S6 completely abolished grayanotoxin-induced effects on Nav1.4 channel. However, the same mutation did not alter BTX binding (Kimura et al. 2000). Together, it can be concluded that the GTX binding site is not completely identical to but is overlapping with the BTX binding site, as they share many molecular determinants.

In contrast with BTX, the channel modification by veratridine (VTD) is less understood. In general, it is assumed that VTD binds to the same site as BTX since VTD induces channel alterations that are similar to these of BTX (Wang and Wang 2003). However, in contrast with BTX, which does not dissociate from its receptor, the binding of VTD to receptor site is reversible, and it dissociates from its receptor upon membrane hyperpolarization (Ulbricht 1998). A bell-shaped relationship was described between the concentration of veratridine and the peak amplitude of sodium current in murine myocytes. It was also observed that increasing concentrations of VTD enhance the peak amplitude, reaching a maximum around 10 μM, while higher concentrations of VTD reduced the sodium conductance (Zhu et al. 2009a). The modulatory effects of aconitine are still poorly studied. It is found that aconitine binding to its receptor causes an incompletion of inactivation and an alteration of the ion selectivity in muscle VGSCs but not in nerve fibers (Campbell 1982).

4 Toxins Binding to Site 3 of VGSCs

Site 3 neurotoxins, including scorpion α-toxins and sea anemone toxins, slow down the inactivation and induce a prolonged opening of VGSCs. According to pharmacological binding properties, scorpion α-toxin can be divided into three groups: α-toxin, α-insect toxin, and α-like toxin. The representative toxins for these three groups include AaH II and Lqh 2, LqhαIT, and Lqh 3 and BmK I, respectively. AaH II has the most potent affinity to site 3 of mammalian neural (rNav1.1, rNav1.2) and muscular (rNav1.1–1.4) sodium channels (Legros et al. 2005). In voltage-clamp experiments in *Xenopus* oocytes, Aah II inhibited the inactivation of the activated rat brain type rNav1.2 and rat skeletal muscle type rNav1.4 and enhanced the Na^+ entry into excitable cells. EC(50) value of Aah II that induced slowing of inactivation is 2.6 ± 0.3 nM on rNav1.2 and 2.2 ± 0.2 nM on rNav1.4 (Alami et al. 2003). The receptor site of AaH II consists of the extracellular loops of DI/S5–S6, DIV/S3–S4, and DIV/S5–S6. A negatively charged amino acid residue (E1613 in rNav1.2, D1428 in rNav1.4) in the loop of DIV/S3–S4 has been shown to play a critical role in the binding of channels with toxins (Cestele and Catterall 2000; Rogers et al. 1996; Leipold et al. 2004). The crystal structure of Aah II has been elucidated, and the structure has been used as template and model for α-scorpion toxin (Gur et al. 2011; Gurevitz 2012).

Lqh αIT, a typical α-insect toxin, is 2,000-fold less active in the mouse brain than Lqh 2 which is a classical α mammal toxin (Gilles et al. 2001). Brain-type sodium channels Nav1.1–3 and Nav1.6 are weakly sensitive to Lqh αIT (Eitan et al. 1990). However, 20 nM of Lqh αIT potently impaired the inactivation of wild-type $Na_v1.4$ channels (Chen et al. 2000). Lqh αIT also interacts strongly with hNav1.7. Residue-swap analysis verified that two acidic residues (Asp1428 and Lys1432 in Nav1.4) within the domain IV S3–S4 extracellular loop of VGSCs were crucial for the selectivity and modulation pattern of Lqh αIT (Leipold et al. 2004). BmK I discriminates well for the three neuronal VGSCs that were independently expressed in *Xenopus* oocytes with the auxiliary β1 subunit: Nav1.6α/β1 responded with a large increase of both transient and persistent currents through inhibition of fast inactivation of sodium channels. Moreover, BmK I also accelerated the slow inactivation and delayed recovery through binding to $Na_v1.6$α/β1 in the open state. Nav1.2α/β1 was less affected by BmK I. At a high concentration (500 nM), BmK I increased fast time constants of inactivating current. In contrast, Nav1.3α/β1 was nearly insensitive (He et al. 2010). Residue-swap analysis verified that an acidic residue (e.g., Asp1602 in $mNa_v1.6$) within the domain IV S3–S4 extracellular loop of VGSCs was crucial for the selectivity and modulation pattern of BmK I, which was in agreement with other α-scorpion toxins binding to sodium channels (Zuo and Ji 2004; Bosmans and Tytgat 2007). Recently, studies showed that expression of Nav1.8 and Nav1.6 were increased in BmK I-induced pain model, indicating a potential pharmacological role of BmK I as a tool to investigate sodium channel-related pain (Ye et al. 2016).

Site 3 sea anemone toxin ATX II enhances the inward currents of Nav1.1, Nav1.2, Nav1.3, Nav1.4, Nav1.5, Nav1.6, and Nav1.7 mainly through increasing the slow component of inactivating currents (Oliveira et al. 2004; Hampl et al. 2016). In addition, ATX produces small persistent currents in Nav1.3 and Nav1.6 at high concentrations (Oliveira et al. 2004). Studies of site-directed mutagenesis found that ATX II interacts with Glu1613 on the extracellular loop of DIV/S3–S4 of the rat neuronal channel Nav1.2 (Rogers et al. 1996). Electrophysiological studies have shown that α-scorpion toxin could bind to rNav1.7 (Cestele et al. 1999), but had no effect on hNav1.7. Sequence comparison reveals that rNav1.7 and hNav1.7 are almost identical in IVS3–S4, with the exception of the acidic residue that is critical for α-toxin binding (Glu in the former vs Asp in the latter). This result also indicated that ATX II and α-scorpion toxin are bound to the site 3 of sodium channel in a different manner.

5 Toxins Binding to Site 4 of VGSCs

The receptor site 4 of voltage-gated sodium channels is mainly recognized by scorpion β-toxins, which exert their activities by altering the gating properties of VGSCs. The major effects of site 4 toxins are to reduce the amplitude of the peak Na^+ currents as well as shift the voltage-dependent activation of VGSCs to more negative potentials (Cestele et al. 2001, 2006; Stevens et al. 2011). Generally, most

of these toxins initiate their modulatory effects on the VGSC activation requiring a short depolarizing prepulse (Leipold et al. 2012). Several key residues, in the extracellular loops connecting S1–S2 and S3–S4 segments of domain II, are important to the receptor site 4 (Catterall et al. 2007). Moreover, it was reported that some residues in the pore region of domain III are critical for β-toxin, such as Tz1 (*Tityus zulianus*) and BmK IT2 (*Buthus martensii* Karsch), to associate with different Na^+ channel isoforms (Leipold et al. 2006; He et al. 2011). One possible allosteric mechanism was that the voltage sensor of domain III enhances the binding of β-toxins to S4 segment in domain II (Song et al. 2011).

Site 4 toxins are always long-chain polypeptides composed of 58–76 amino acid residues with cross-linked by four disulfide bridges which belong to Csα/β motif structural superfamily (de la Vega and Possani 2007; Gurevitz et al. 2007; Possani et al. 1999). β-scorpion toxins could be divided into four groups according to their affinity and pharmacological characterizations to mammalian or insect VGSCs – classical (mammalian-selective) β-toxins, excitatory anti-insect β-toxins, depressant anti-insect β-toxins, and β-like (acting on both mammalians and insects) toxins.

1. Classical β-toxins: Classical β-toxins show high affinity to mammal Na^+ channels (Martin et al. 1987; Pintar et al. 1999). Examples of classical β-toxins include Css4 (*Centruroides suffusus suffusus*) and Cn2 (*Centruroides noxius* Hoffmann).
2. Excitatory anti-insect β-toxins: Excitatory anti-insect β-toxins fail to show any effects on mammal channels (de Dianous et al. 1987), whereas they induce a fast and repetitive activity of motor nerves by targeting insect VGSCs that results in a reversible contraction paralysis. AahIT (*Androctonus australis* Hector), BmK IT (*Buthus martensii* Karsch), LqqIT1 (*Leiurus quinquestriatus quinquestriatus*), and Bj-xtrIT (*Hottentotta judaicus*) belong to this group (Liu et al. 2011; Froy et al. 1999; Billen et al. 2008; Ji et al. 1994). Compared to other β-toxin groups, one disulfide bridge of excitatory anti-insect β-toxins is located differently, which results in different secondary structural elements. The high selectivity to insect but not mammal, combined with high potency, makes the excitatory anti-insect β-toxins promising lead compounds in the design of new insecticides (Gurevitz et al. 2007).
3. Depressant anti-insect β-toxins: These toxins induce a transient muscle contraction and followed by a slow depressant as well as flaccid paralysis (Zlotkin et al. 1991; Karbat et al. 2007). Representatives of this group are LqqIT2 (*L. q. quinquestriatus*), BjIT2 (*H. judaicus*), and BmK IT2 (*Buthus martensii* Karsch). Current-clamp experiments showed that the evoked action potentials were suppressed by toxins in this group (Strugatsky et al. 2005; Li et al. 2000). Insect-selective depressant β-toxins can also modulate mammalian VGSCs under certain conditions such as when mammalian VGSCs are excited by a long depolarizing prepulse or when there is a simultaneous binding of an α-toxin to site 3 (Cohen et al. 2007a). Furthermore, BmK IT2, a depressant anti-insect β-toxin from *Buthus martensii* Karsch, inhibited the currents of mammalian

VGSCs in DRG (dorsal root ganglion) neurons as well as hippocampal pyramidal neurons without a long depolarizing prepulse or a simultaneous binding of α-toxin to site 3 (Zhao et al. 2008; Tan et al. 2001). Combinational site 3 α-toxin (BmK I) and BmK IT2 resulted in larger peak I_{Na} and more negative half-activation voltage compared to BmK I alone. Co-applied BmK I and BmK IT2 also produced slower inactivation compared to BmK I alone (Feng et al. 2015). These results suggested that existence of depressant β-toxins in the scorpion venom may contribute to the scorpion venom-induced toxicity through enhancing effects of α-toxins.

4. β-like toxins: β-like toxins are capable of competing for binding sites on both insect and mammalian Nav channels. Tsγ (*Tityus serrulatus*), Lqhβ1 (*Leiurus quinquestriatus hebraeus*), and BmK AS (*Buthus martensii* Karsch) are well-studied examples of β-toxins (Possani et al. 1999; Ji et al. 1999; Gordon and Gurevitz 2003). Interestingly, BmK AS exhibits the pharmacological activities of both the α- and β-toxins, which could not only hyperpolarize the voltage dependence of activation but also delay the slow inactivation of VGSCs (Zhu et al. 2009b).

A bioactive surface of β-scorpion toxins is composed of two main clusters. The first cluster is associated with the α-helix, and it contains a conserved negatively charged residue, Glu24 in LqhIT2, Glu26 in Lqhβ1, Glu28 in Css4, and Glu30 in Bj-xtrIT. The conserved residue is highly important for the toxin activity and probably interacts with a positively charged counterpart of VGSC. The residues flanking the glutamate are hydrophobic and seem to form a hydrophobic environment that occludes bulk solvent from the high-energy point of interaction. The common structure of the cluster could explain the ability of β-toxins to compete for receptor 4 of different neuronal membrane preparations (Gurevitz et al. 2007). Cn2, a classical anti-mammalian β-toxin, could compete with excitatory toxin Bj-xtrIT on binding to insect VGSCs (Cohen et al. 2004). BmK IT2 shifted the activation of DmNav1, the sodium channel from *Drosophila*, to more hyperpolarized potentials, whereas it hardly affected the gating properties of rNav 1.2, rNav 1.3, and mNav 1.6, three mammalian central neuronal sodium channel subtypes. However, for DRG neurons, BmK IT2 potently blocked both tetrodotoxin-sensitive (TTX-S) and tetrodotoxin-resistant (TTX-R) components (He et al. 2011; Tan et al. 2001). The second cluster of the bioactive surface of β-scorpion toxins is composed of hydrophobic residues including Leu19, Asn22, Tyr40, Tyr42, and Phe44 in Css4, Val71, Gln72, Ile73, and Ile74 in Bj-xtrIT. These residues are associated mainly with the β2 and β3 strands and are likely to confer the specificity of β-toxins for VGSCs (Cohen et al. 2005). The commonality of the pharmacophore at the βαββ core of β-toxins leads to an attempt to evaluate the contribution of the pharmacophore to the binding affinity of β-toxins (Cohen et al. 2004, 2005). Removal of the N- and C-terminal regions in Bj-xtrIT and Css4 resulted in truncated toxin derivatives that were successfully folded in vitro and examined in binding competition assays. However, the truncated toxins did not bind at receptor site 4 nor compete with the parental toxins for this site. Unexpectedly, they were able to modify

allosterically the binding and activity of site 3 scorpion toxins (anti-mammalian Lqh2 and anti-insect LqhaIT (Cohen et al. 2008).

The pharmacological properties of VGSC receptor site 4 have been well investigated and defined in more than two decades (Gurevitz et al. 2007; Cohen et al. 2006). By analyzing the bioactivity of the β-toxin Ts1 from *T. serrulatus* on chimeras made among brain, cardiac, and skeletal muscle VGSCs, domain II has been found to be important for toxin binding (Marcotte et al. 1997). This conclusion was also supported by mutation and domain substitution studies of Css4 and AahIT, respectively. Substitution of Glu779 at DII/S1–S2 or substitution of Glu837, Leu840, and Gly845 at DII/S3–S4 significantly reduced the bioactivity of Css4 on rNav1.2a (Cestele et al. 1998). Substitution of domain II in rNav1.2a with the equivalent domain from the *Drosophila* channel DmNav1 converted the insensitivity to sensitivity of AahIT (from *Androctonus australis* Hector) to rNav1.2a (Shichor et al. 2002). Based on these results, Cestèle's group constructed a model of site 4 toxins docking at the "gating module" of rNav1.2a (Cestele et al. 2006). Though the structure model described a putative face of interaction between Css4 and the rNav1.2a channel in the "gating module" of domain II, the region of VGSCs that binds the toxin surface involved in selectivity was proposed to be associated with the "pore module." Substitutions at the "pore module" of domain III (domain III SS2–S6) on rNav1.4 highlighted the involvement of Glu1251 as well as His1257 in the recognition on the β-toxin Tz1 (Leipold et al. 2006). In order to discriminate the key residues in rNav1.4, Cohen's group (Cohen et al. 2007b) employed Css4 mutants in double-mutant analysis. Close proximity between the pairs was suggested by DDG values: F14A at the toxin and E592A at domain II S1–S2 of the channel, R27Q at the toxin and E1251N at domain III SS2–S6 of the channel, and E28R at the toxin with both E650A at domain II S3–S4 and E1251N at domain III SS2–S6 of the channel. The above results suggested that receptor site 4 contains mainly the domains II and III of VGSCs. A crevice between extracellular linkers S1–S2 and S3–S4 at the gating module of domain II might be involved (Gurevitz 2012; Karbat et al. 2004). Moreover, BmK IT2 from *Buthus martensii* Karsch strongly shifted the activation of DmNav1, the sodium channel from *Drosophila*, to more hyperpolarized potentials, whereas it hardly affected the gating properties of rNav1.2a, rNav1.3, and mNav1.6, three mammalian central neuronal sodium channel subtypes. Mutations of Glu896, Leu899, and Gly904 in extracellular loop domain II S3–S4 of DmNav1 abolished the functional action of BmK IT2. In addition to domain II, Ile1529 in domain III pore loop was critical for recognition and binding of BmK IT2 (He et al. 2011). The results provided the support to the voltage sensor-trapping mechanism and offered novel insights into the molecular requirements for the development of toxin modulators.

Similar to scorpions, spiders are capable of producing neurotoxins, recognizing on the receptor site 4 of VGSCs. It is known to all that Magi 5 (*Macrothele gigas*) is the first spider toxin shown to compete with the scorpion β-toxin CssIV for receptor site 4 (Corzo et al. 2003). Other site 4 spider β-toxins include δ-palutoxins (*P. luctuosus*), curtatoxins (*Hololena curta*), μ-agatoxins (*Agelenopsis aperta*), β/δ-agatoxins (*A. orientalis*), and the recently characterized Jingzhaotoxins (*Chilobrachys jingzhao*)

as well as Huwentoxins (*Ornithoctonus huwena*) (Stapleton et al. 1990; Corzo et al. 2000; Billen et al. 2010; Rong et al. 2011; Tang et al. 2014; Wei et al. 2014; Xiao et al. 2008). Spider β-toxins are composed of 34–37 residues cross-linked by four disulfide bridges, which forms an ICK (inhibitor cysteine knot) motif (Nicholson 2007). It was found that the μ-agatoxins shifted the voltage activation curve to more hyperpolarized potentials and slowed down the inactivation process of VGSCs, resulting in a non-inactivating persistent current (Adams 2004), like scorpion β-toxin BmK AS (Zhu et al. 2009b). β/δ-Agatoxins caused a bell-shaped voltage-dependent modulation on both the activation and inactivation of VGSCs (Billen et al. 2010). Interestingly, δ-palutoxins, which compete with the depressant scorpion β-toxin Bj-xtrIT for site 4, actually functioned as α-toxins by slowing down the inactivation of VGSC (Corzo et al. 2000). However, these toxins fail to displace the binding of α-toxin LqhαIT (Corzo et al. 2005). Huwentoxin-IV (HWTX-IV), a 35-residue peptide from tarantula *Ornithoctonus huwena* venom, selectively inhibits neuronal VGSC subtypes rNav1.2, rNav1.3, and hNav1.7 compared to muscle subtypes rNav1.4 and hNav1.5. The toxin docked at receptor site 4 located at the extracellular S3–S4 linker of domain II. Mutations E818Q and D816N in hNav1.7 decreased toxin affinity, whereas the reverse mutations in rNav1.4 (N655D/Q657E) and the corresponding mutations in hNav1.5 (R812D/S814E) greatly increased the sensitivity of the muscle VGSCs to HWTX-IV (Xiao et al. 2008). Jingzhaotoxin-III (JZTX-III), a well-studied 36-residue peptide from the tarantula *Chilobrachys jingzhao*, inhibited Nav1.5. It traps the DII voltage sensor of Nav1.5 by binding to the DIIS3–S4 linker (Rong et al. 2011). Two acidic residues (Asp1, Glu3) as well as four Trp residues (residues 8, 9, 28, and 30) play crucial roles in the binding of JZTX-III to Nav1.5. Mutations S799A, R800A, and L804A could additively reduce toxin sensitivity of Nav1.5. Arg800, not existent in other sodium channel subtypes, is responsible for the selective interaction of JZTX-III with Nav1.5 (Rong et al. 2011). In addition, Jingzhaotoxin-XI (JZTX-XI, *Chilobrachys jingzhao*) is also specific for Nav1.5, inhibiting the sodium conductance as well as slowing the fast inactivation (Tang et al. 2014). In conclusion, the results suggested that spider β-toxins probably have additional contact points with the extracellular loops of VGSCs, besides receptor site 4.

6 Toxins Binding to Site 5 of VGSCs

Ciguatoxins (CTX, *Gambierdiscus toxicus*) and brevetoxins (BTX, *Karenia brevis*) are highly lipophilic cyclic polyether compounds that are biosynthesized by the worldwide distributed epibenthic and planktonic dinoflagellates. The physiological and pathological, especially neurological, effects of both CTXs and BTXs are the results of their interaction with the receptor site 5 of VGSCs (Lombet et al. 1987; Rashid et al. 2013; Pearn 2001). Brevetoxins (PbTxs) consist of 10–11 transfused rings, 23 stereocenters, and an overall linear low-energy conformation (Cassell et al. 2015; Turner et al. 2015). So far, at least 14 brevetoxins have been identified. PbTx-1 and PbTx-2 are two most classical brevetoxins and the parent toxins. The

parent toxins PbTx-1 and PbTx-2 are different from each other in their backbone structure (type A and type B) (Cassell et al. 2015; Turner et al. 2015). Type A includes the PbTxs 1, 7, and 10, whereas type B includes PbTxs 2, 3, 5, 8, and 9. All PbTxs possess a lactone in the A ring as well as a conservative rigid region that forms a ladder structure, which is separated from the A ring by a spacer region with limited flexibility (Gawley et al. 1995). Moreover, they all possess a side chain that allows modification at the termini of molecules (Baden et al. 2005). It is believed that the terminal, rigid four-ring system is involved in channel binding, while the functional lactone A-ring is responsible for the alterations in channel inactivation and prolongation of the mean open time (Jeglitsch et al. 1998; Purkerson-Parker et al. 2000). PbTxs interact with VGSCs by intercalating in the membrane in a head-down orientation. Previous studies have indicated that the toxins position themselves across the plasma membrane, which is parallel with the transmembrane segments, with the A ring toward the intracellular side as well as the tail terminal of the molecule facing the extracellular side (Jeglitsch et al. 1998; Trainer et al. 1994). However, the key residues involved in brevetoxin activity still remain unknown. PbTx binding at site 5 leads to distinct alterations in channel gating: (1) the activation potential is shifted toward hyperpolarized potentials; (2) channels remain longer in the open configuration which results in a longer mean open time; (3) the inactivation is slowed down or inhibited; and (4) brevetoxins have, among all known voltage-gated sodium channel modifying toxins, the unique capability to stabilize more than one conductance levels. As such brevetoxin binding induces distinct sodium ion subconductance states in addition to the normal 21 pS state (Baden et al. 2005; Jeglitsch et al. 1998; Schreibmayer and Jeglitsch 1992). Moreover, it was found that the S6 of DI and S5 of DIV participate in the formation of receptor 5 of VGSCs using a photoreactive PbTx-3 derivate as a probe (Trainer et al. 1994). $^{[3H]}$PbTx-3 is specifically bound to Nav1.4, or the Nav1.5 α subunit isoforms expressed in HEK cells. Nav1.5 appeared to be less sensitive to BTXs compared to Nav1.4. Both type A (PbTx-1) and type B (PbTx-2 as well as PbTx-3) PbTxs target both cardiac and muscle channels. Type B PbTxs exhibit a lower affinity for the heart compared to the skeletal muscle channel (Bottein Dechraoui and Ramsdell 2003). The evaluation of the relative affinity of PbTx-2 and PbTx-3 as well as CTX with VGSCs was studied by competitive binding in the presence of $^{[3H]}$PbTx-3 in the brain, heart, and skeletal muscle of rat and the marine teleost fish *Centropristis striata*. No significant differences between the rat and fish were observed in the binding of PbTxs and CTX to either the brain or skeletal muscle. However, $^{[3H]}$PbTx-3 showed a substantially lower affinity for rat heart tissue compared to fish heart tissue and other tissues.

Ciguatoxins (CTXs) are, similar to brevetoxins, lipid-soluble compounds, which are composed of 13 ether rings with a structural backbone (Yasumoto 2001). Though 29 ciguatoxin derivates have been identified, their biological activity information remains unclear, which is mainly due to difficulties in obtaining pure toxins (Perez et al. 2011). Competing with brevetoxins for site 5, CTXs induce similar modifications of channel gating. CTXs could shift the activation of VGSCs toward more negative potentials as well as inhibit the fast inactivation. Even though

binding at the same receptor site, PbTxs and CTXs possibly differ from each other in their mechanism of action. CTXs were capable of producing VGSCs dependent oscillations in neuronal membrane potential (Hogg et al. 2002). Recently, it was reported that CTXs cause a concentration-dependent decrease of the amplitude of Na^+ currents in sensory neurons (Cohen et al. 2008; Perez et al. 2011; Yamaoka et al. 2009). The most powerful ciguatoxin P-CTX-1 has attracted the most attention from researchers. P-CTX-1 binds to native channels in the brain, heart, and skeletal muscle of the rat as well as the marine teleosts in the presence of $^{[3H]}$PbTx-3 (Shmukler and Nikishin 2017; Dechraoui et al. 2006). An exhausting study of the electrophysiological effects of P-CTX-1 on VGSC isoforms Nav1.1–1.9 was carried out in HEK293 cells. P-CTX-1 has shown the ability to influence all Nav isoforms. P-CTX-1 decreases the activation threshold of two key isoforms, Nav1.7 as well as Nav1.8 channels, and lengthens their active periods, contributing to the increased activity of nociceptive neurons (Inserra et al. 2017).

7 Toxins Binding to Site 6 of VGSCs

Among all sites, neurotoxin receptor site 6 is still the speculative and yet undefined site. A first proposal for this site arose when TxVIA, one kind of δ-conotoxins (δ-CTXs), was characterized from the cone snail *Conus textile*. The polypeptide toxin TxVIA has led to the identification of neurotoxin receptor site 6 on sodium channels. TxVIA causes a marked prolongation of action potentials, due to specific inhibition of sodium current inactivation (Hasson et al. 1993). So far, 19 δ-CTX sequences (such as GmVIA, PVIA, NgVIA, SVIE, and EVIA) have been identified in fish and mollusk-hunting cone snails. The δ-CTXs act at site 6 composed of a set of amino acid residues in the segment 4 of domain IV and slow down sodium channel inactivation (Fainzilber et al. 1994). The mechanism of binding and action is quite different between δ-CTXs and μ-CTXs. For example, δ-CTXs only target neuronal voltage-gated sodium channels. δ-CTXs do not reduce channel conductance associated with pore-blocking toxins. Instead δ-CTXs affect the kinetics of the channel by reducing inactivation and thus prolonging the time that the channel remains open (Fainzilber et al. 1995). In addition, δ-CTXs exhibit synergistic effect with α-scorpion toxins, probably because both of these toxins trap the IVS4 voltage sensor in a similar conformation. However, the two toxins clearly do not directly compete for the same binding site at the voltage sensor. Instead, they apparently stabilize each other at their target sites such that these toxins affect the gating of sodium channels in a highly synergistic manner, which hints that the δ-CTXs may slow inactivation by the same molecular mechanism as α-neurotoxins from scorpion, sea anemone, spider, etc. (Leipold et al. 2005).

8 Conclusion

VGSCs are important in physiology and pathology of multiple tissues. In recent years, VGSCs has been serving as promising drug development targets for multiple diseases. However, either small molecule inhibitors or monoclonal antibodies of VGSCs have not been successful in developing novel therapeutic drugs. Natural toxins targeting VGSCs provide unique tools for studying molecular structure and function, pharmacology, and drug development potential of VGSCs. With the increasing understanding of detailed mechanism of VGSC-toxin interaction, combined with development of drug-developing techniques, VGSC-targeting toxins and their functional domains could be used as the lead molecules or part of the lead molecules for the successful development of therapeutic drugs in multiple diseases in the future.

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