Review

Targeting voltage sensors in sodium channels with spider toxins

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Voltage-activated sodium (Nav) channels are essential in generating and propagating nerve impulses, placing them amongst the most widely targeted ion channels by toxins from venomous organisms. An increasing number of spider toxins have been shown to interfere with the voltage-driven activation process of mammalian Nav channels, possibly by interacting with one or more of their voltage sensors. This review focuses on our existing knowledge of the mechanism by which spider toxins affect Nav channel gating and the possible applications of these toxins in the drug discovery process.

Introduction

Voltage-activated sodium (Nav) channels are Na^+-permeable ion channels that open and close in response to changes in membrane voltage, and primarily contribute to the rising phase of the action potential [1,2]. In humans, the medical relevance of Nav channels is reflected by mutations that underlie debilitating disorders such as cardiac arrhythmias, epilepsy, muscle weakness, and erythermalgia [3–6] . In extreme cases, abnormalities in Nav channels can even eliminate the ability to feel pain, enabling a person to walk on burning coals [7]. Nav channels are therefore considered to be important therapeutic targets, yet our appreciation of these channels is hampered by a lack of insight into their complex structure and mechanism of action.

Nine mammalian isoforms of Nav channels have been identified (1.1–1.9) [8]. They share a similar (but complex) architecture. The principal channel-forming α-subunit consists of four homologous domains (I–IV), each containing six transmembrane segments (S1–S6). The S5–S6 segments from the four domains collectively form the central ion conduction pore for Na^+, with the S1–S4 segments from each domain forming the surrounding voltage sensors (Figure 1a and b). In a few subtypes in which it has been examined (e.g. Nav1.2, Nav1.4 and Nav1.5), each of the four voltage sensors activate in response to changes in membrane voltage. However, those in domains I–III are the most important for channel opening, whereas the one in domain IV plays a unique part in inactivating the channel only milliseconds after channel opening [9–12]. It remains to be seen if this *modus operandi* applies to all subtypes of Nav channels or whether the voltage sensors in more distantly related Nav channels (e.g. Nav1.8 and Nav1.9) have distinct operational mechanisms.

Nav channels are one of the foremost targets of molecules present in animal venoms [13] . Toxins from scorpions and sea anemones, as well as venoms from cone snails, have been used to describe various receptor sites in different regions of the channel [14–17]. However, the exploration of the mechanism through which spider toxins interact with mammalian Nav channels has only recently begun. This is in contrast to voltage-activated potassium (Kv) channels, where tarantula toxins such as hanatoxin from *Grammostola spatulata* have been used extensively to study the functional properties of these channels [18–21]. Evidence demonstrating that tarantula toxins modify the opening and closing ('gating') of Kv channels by influencing their voltage sensors comes from three observations. First, toxin-bound channels can still open and conduct ions, but the energy required to open toxin-bound channels is typically increased [22–24]. Second, mutagenesis experiments suggest that the toxins interact with defined regions within the voltage sensors [21,25,26]. Third, these toxins have distinct effects on voltage sensor movements, as reflected in gating current measurements [27]. (Charged arginine residues in the S4 segment of the voltage sensor move in response to a change in membrane voltage, and this movement can be detected as a non-linear capacitive current or gating current [28].)

ProTx-I and ProTx-II from *Thrixopelma pruriens* are two of the better-studied toxins that modify gating of mammalian Nav channels [12,29–34]. These tarantula toxins are closely related to hanatoxin (Figure 1c) and appear to work through similar mechanisms (see below), which fits nicely with the recent discovery that hanatoxin can also inhibit Nav channels at concentrations similar to those that modify the gating of Kv channels [12]. Several spider toxins with related amino-acid sequences have now been identified (Figure 1c), many of which interact with Nav channels and modify gating through what appears to be three distinct mechanisms. The first, and most commonly observed, is for the toxin to inhibit opening of the channel in response to membrane depolarization [12,29–31,33,34], as illustrated in Figure 2a for ProTx-I. A second mechanism is for the toxin to hinder fast inactivation, as observed for SGTx1 from the *Scodra griseipes* tarantula (Figure 2a) and JzTx-IV from the *Chilobrachys jingzhao* tarantula [35]. A third, as observed in the case of Magi5 from the hexathelid spider *Macrothele gigas*, is for the

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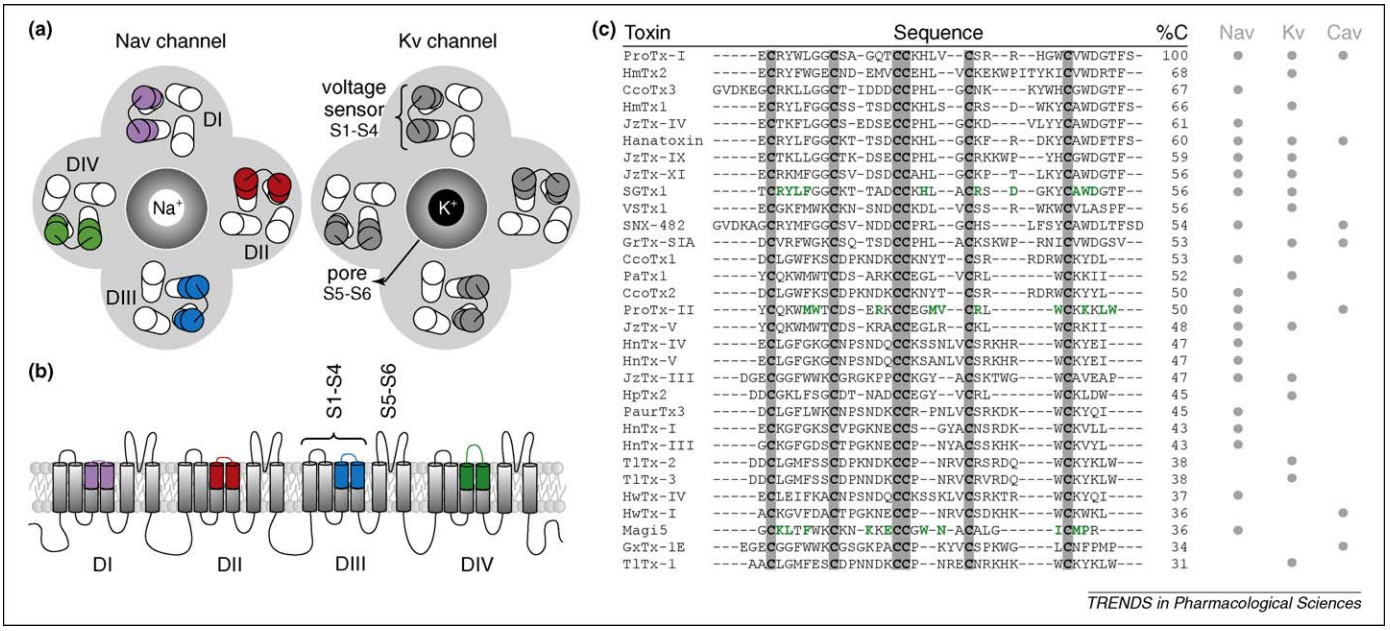

Figure 1. Spider toxins that target voltage-activated ion channels
(a) Schematic representation of the top view of a Nav channel (left) and Kv channel (right). The central Na^+- or K^+-selective pore is surrounded by the four voltage sensors of the four domains (DI–DIV). In the Nav channel, the paddles are not identical and are therefore colored differently. In the Kv channel, the paddles are identical and therefore have the same color. **(b)** Side view of a Nav channel imbedded in a lipid membrane. Each domain (DI–DIV) consists of six transmembrane segments (S1–S6) of which S1–S4 form the voltage sensor and the S5–S6 segments of each domain come together to form the Na^+-selective pore of the channel. **(c)** Sequence alignment of spider toxins with an ICK motif and three disulfide bridges that target Nav, Kv, and/or Cav channels (indicated by gray circles). Residues that have been shown to be a part of the functionally important surfaces of SGTx1 [38], ProTx-II [33], and Magi5 [36] are indicated in green; %C = % conserved residues.

toxin to facilitate opening of the channel by shifting activation of Nav channels to more hyperpolarized voltages [36] (Figure 2a). Although these disparate effects of Nav channel toxins seem complex, they can be understood in conceptually simple terms if one considers the voltage sensors they target and how those sensors couple to various aspects of Nav channel gating. We will review what is known about the interaction of spider toxins with the four voltage sensors in Nav channels, the emerging role of the lipid membrane in determining the pharmacology of Nav channels, and the potential medicinal applications of these toxins .

Structural features of Nav channel voltage-sensor toxins

Most spider toxins targeting mammalian Nav channels consist of 30–40 amino acids. The core of the molecule is stabilized by 3–4 disulfide bridges. The inhibitory cystine knot (ICK) motif [37] seems to be the most commonly used fold, but does not specify the mechanism of action because some ICK toxins act as pore blockers and others as gating-modifiers [20]. Studies on tarantula toxins interacting with voltage sensors in Kv channels have provided a comprehensive map of their functionally important surfaces. For example, alanine scanning mutagenesis of SGTx1 has revealed that the active face of the molecule consists of a hydrophobic protrusion surrounded by a ring-like assembly of highly polar residues [38] (Figure 2b). The affinity of SGTx1 for Nav channels is even higher than for Kv channels [12], so these structural features are probably important for most spider toxins interacting with Nav channels. Indeed, amphipatic structures are found in a range of spider toxins that interact with voltage sensors in Nav channels (e.g. hanatoxin [39], ProTx-II [29], PaurTx3 [40], CcoTx1 [40], and HwTx-IV [41]) and Kv channels (e.g. hanatoxin [39], VSTx1 [42], PaTx1 [43], and HpTx2 [44]).

Toxins having seemingly distinct effects on Nav channel gating (Figure 2a) are similar when comparing functionally important surfaces. The nuclear magnetic resonance (NMR) structures of SGTx1 [38] and Magi5 [36] show that both toxins contain a cluster of hydrophobic residues surrounded by basic, acidic and other highly polar residues (Figure 2b). Although the overall fold and amphipatic nature of the functionally important surfaces are similar, there are subtle differences in the residues that are crucial for activity. The active face of SGTx1 towards Kv channels contains the solvent-exposed hydrophobic surface in which mutations of L5, F6 and W30 result in dramatic weakening of toxin affinity. Of the polar residues, the positively charged R3 and R22 greatly decrease toxin affinity, probably by removing electrostatic interactions with polar residues within the voltage sensor. In Magi5, it seems that F6, W7, I25, M27 and P28, which are all on the hydrophobic face of the molecule, are essential for toxin activity towards Nav channels. The polar residues K10, E14 and N19 greatly reduce toxin effects on channel activation if they are replaced with alanine. Interestingly, Magi5 resembles the surface of the differently folded β-scorpion toxin CssIV [36]. Both of these toxins cause Nav channels to open at more negative voltages, and competition studies suggest an interaction with a common receptor site [45]. Previous work on CssIV revealed an important role for the negatively charged E15 in stabilizing the voltage sensor of domain II in an activated state, thereby promoting channel activation [46]. Remarkably, E14 in Magi5 is also essential for toxin affinity, and may therefore enable it to affect channel activation through the same mechanism [45].

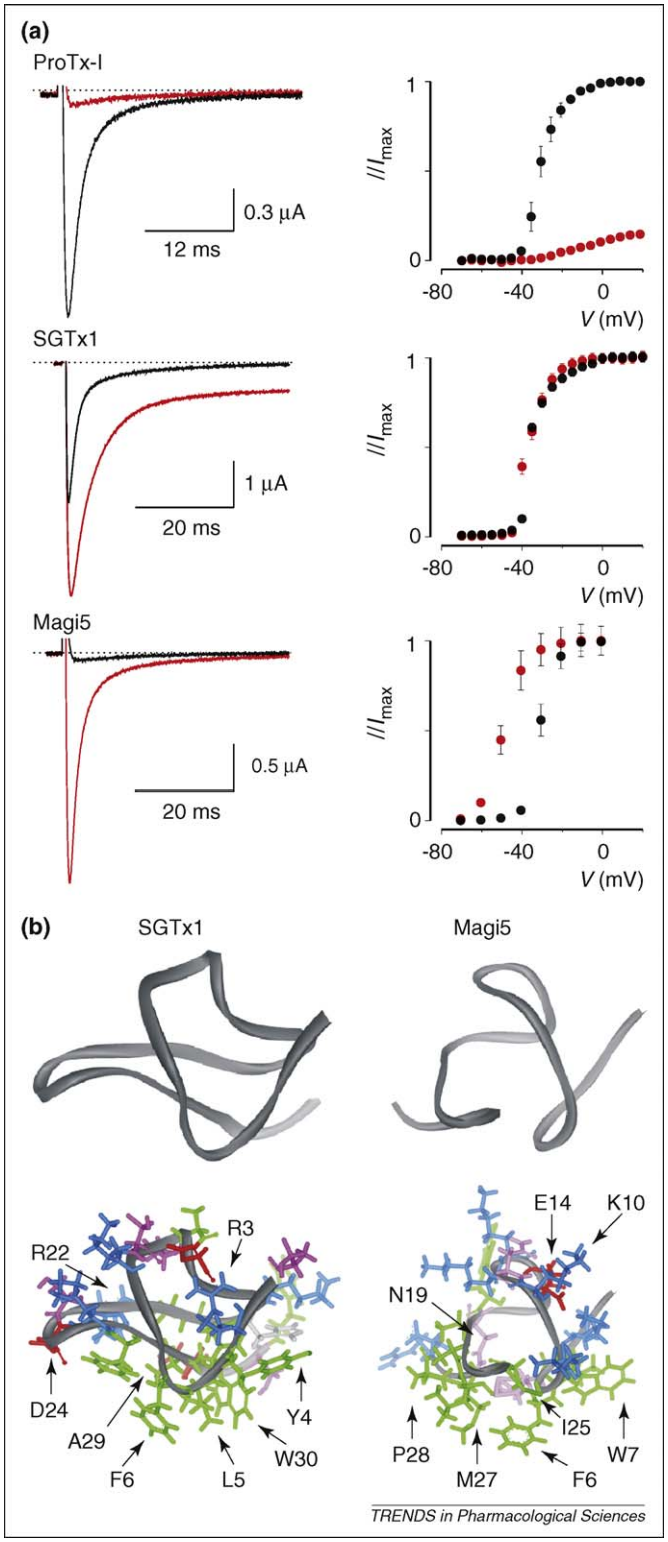

Figure 2. Interactions between spider toxins and Nav channels
(a) Effects of 100 nM ProTx-I, 100 nM SGTx1, and 1 μM Magi5 on rNav1.2a channels expressed in *Xenopus laevis* oocytes and recorded with the two-electrode voltage-clamp technique. Left, sodium currents elicited by a depolarization to a suitable membrane voltage before (black) and after toxin addition (red) are shown. Right, corresponding conductance–voltage relationships are shown (n = 3; error bars are s.e.m.). **(b)** NMR solution structures of SGTx1 and Magi5. Residue coloring is as follows: blue, basic; red, acidic; green, hydrophobic; white, histidine; pink, serine/threonine/asparagine. Backbone fold is shown on top in dark-gray. Images were created using DSViewer Pro and Protein Data Bank accession IDs 1LA4 for SGTx1 [38] and 2GX1 for Magi5 [36].

Toxin–channel interactions

Classic studies on scorpion venom have established the presence of toxins that interact with the voltage sensors in Nav channels [45,47–51]. In the conventional view, these toxins interact with extracellular loops between S3 and S4 to stabilize the voltage sensors in particular states [45,50,52,53]. In parallel, spider toxins affecting Kv channels were shown to interact with a specific structural motif within the voltage sensors; this motif is composed of S3b and S4 helices, and is also referred to as the 'paddle motif' (Box 1) [21,22,25,26,54–59].

Defining the protein–protein interface between voltage-sensor toxins from spiders and mammalian Nav channels has been a challenge. Initially, it was suggested that the functional similarities between hanatoxin and the protoxins (ProTx-I and ProTx-II) implied a toxin receptor in the S3–S4 region of Nav channels [29]. However, Nav channels have four of such regions, one in each voltage sensor and all have similar amino-acid sequences. Therefore, the authors hypothesized that voltage-sensor toxins from tarantulas might simultaneously interact with different voltage sensors within one Nav channel, but tools to explore this possibility were not yet available. Even extensive mutagenesis and the swapping of S3–S4 regions between Nav1.5 domains by Smith and colleagues did not reveal the binding site of ProTx-II [33].

The first indication of an interaction site and a working mechanism of these tarantula toxins came only recently when the research group of Catterall showed that ProTx-II impedes movement of the gating charges in Nav1.2, thereby providing evidence that this toxin can influence the movement of voltage sensors in Nav channels [34]. The Leu833Cys mutation in the S3–S4 region in domain II reduced affinity for ProTx-II by about twofold, and

Box 1.

Unlike Nav channels, Kv channels are composed of four identical subunits that come together to form a functional voltage-activated K^+ ion-selective channel. Each subunit possesses a voltage-sensing domain (S1–S4) and a region (S5–S6) that forms the central pore domain [55]. The external four arginine (or lysine) residues in S4 are positively charged and carry most of the gating charge, thereby driving conformational changes of the voltage sensor in response to changes in membrane voltage [84–86]. It is thought that all four voltage sensors must activate before the channel opens [87]. Extensive studies on the voltage sensor in Kv channels have identified a specific S3b–S4 helix-turn-helix structural motif (also known as the 'voltage sensor paddle') which moves in contact with the surrounding lipid membrane in response to changes in membrane voltage [21,54–59] Figure I . It was recently shown that paddle motifs are modular units and can be transferred between ion channels and proteins with a voltage-sensing domain without losing their functional properties, suggesting that this motif resides in a relatively unconstrained environment [12,21]. Moreover, the paddle is an important pharmacological target in ion channels because various tarantula toxins were shown to interact with this region [12,24,25,66–68]. Hanatoxin is the founding member of a family of toxins that bind to the paddle motif in Kv channels and inhibit opening of these channels by stabilizing a resting conformation of the voltage sensor [22–24]. Although one hanatoxin molecule can inhibit activation of the Kv channel, it is probable that toxin occupancy of the channel can be as high as four [22,24].

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Box 1. (cont.)

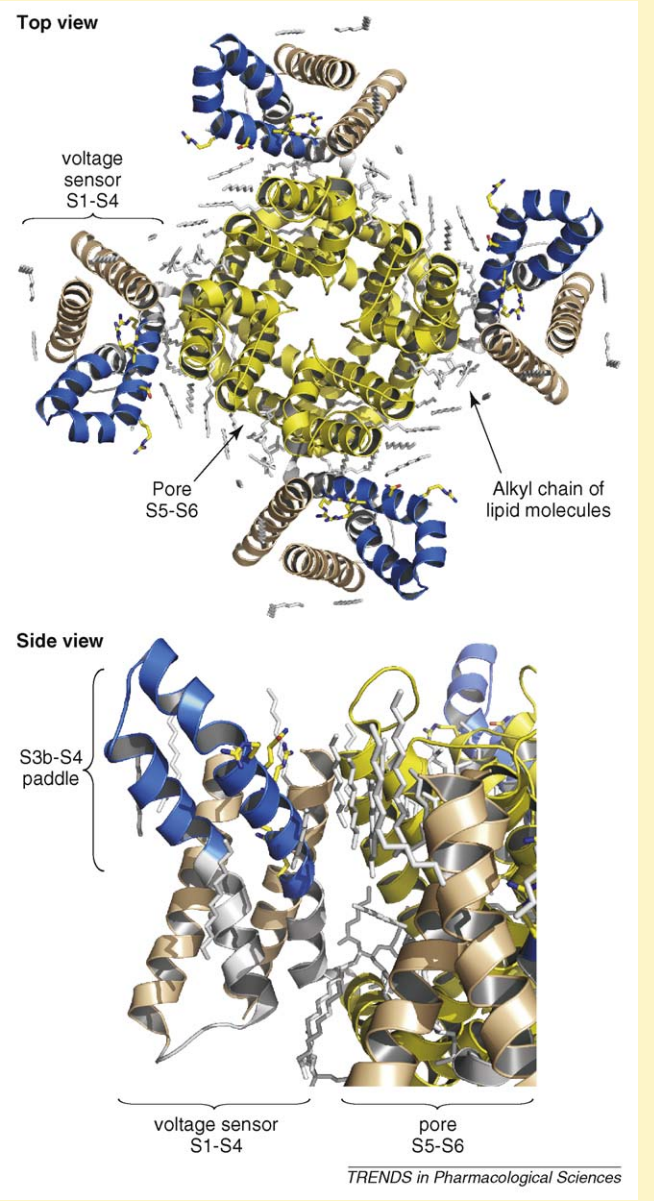

Figure I. Ribbon representation of the X-ray structure of a paddle chimera between the Kv2.1 and Kv1.2 channel viewed from the external side of the membrane (top view) and from within the membrane (side view) [57]. The S3b–S4 paddle motif is colored blue, the pore domain (S5–S6) is colored yellow, and possible lipid molecules are colored gray. Basic residues in S4 are shown as stick representations (Protein Data Bank accession ID is 2R9R). The side view of the chimeric channel shows the S1–S4 voltage-sensing domain and its interface with the pore domain together with the possible location of lipid molecules.

mutation of the outermost two arginines to glutamine weakened voltage-dependent reversal of toxin action and toxin inhibition of gating current. They also reported substantially different inhibition characteristics of ProTx-II between Nav1.2 and Nav1.5, raising the possibility that the toxin may interact with distinct receptors on different Nav channels subtypes. Shortly after this study, two research groups reported that certain amino acids in the domain II voltage sensor of Nav1.7 are involved in the interaction between this particular channel and two voltage sensor toxins: ProTx-II [31] and HwTx-IV [60]. However, mutating equivalent residues in Nav1.2 did not significantly influence sensitivity to ProTx-II, suggesting other interaction sites in different regions of the channel.

A common theme in all of these studies is that the interaction between Nav channels and voltage-sensor toxins from spiders is multifaceted and therefore difficult to define [29,30,33,34]. A significant contribution to our understanding of this interaction was made when S3b–S4 paddle motifs were identified within the four Nav channel voltage sensors and transplanted into fourfold symmetric Kv channels to individually examine their interactions with toxins from tarantulas and scorpions [12]. An advantage of this paddle transfer approach is that it allows individual toxin–paddle interactions to be studied in isolation, eliminating the masking effects of toxins interacting with multiple paddle motifs in Nav channels. In this study, it was demonstrated that the paddle motif in each of the four Nav channel voltage sensors can interact with toxins from tarantulas or scorpions, and that multiple paddle motifs are often targeted by a single toxin. For example, it was shown that ProTx-II can interact with the voltage sensor in domain I, II and IV, whereas ProTx-I interacts only with domain II and IV [12] (Figure 3). It is also interesting that the profiles of toxin–paddle interactions vary for different subtypes of Nav channels, which could help to explain the disparate effects of mutations in Nav1.7 and Nav1.2 on inhibition by ProTx-II [31].

A particularly fascinating problem with toxins targeting voltage sensors is that the effects on Nav channel gating are remarkably diverse. One rule emerging from the paddle transfer approach is that toxins targeting the voltage sensors in domains I–III influence the opening of Nav channels, whereas toxins must specifically interact with the voltage sensor in domain IV to impede inactivation

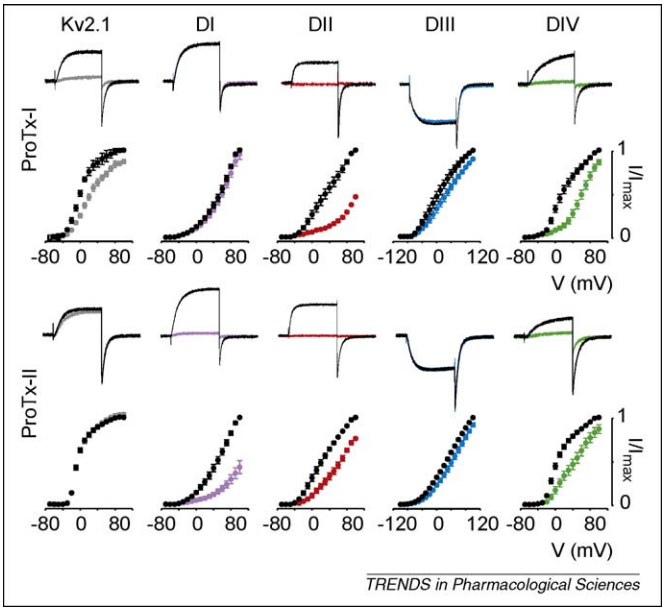

Figure 3. Sensitivity of rNav1.2a paddle chimeras to ProTx-I and ProTx-II
Effects of 100 nM ProTx-I and ProTx-II on Kv2.1 and rNav1.2 paddle chimeras in which paddle motifs were transferred from each voltage-sensing domain from rNav1.2a into Kv2.1 [12] are shown. For each toxin and construct, potassium currents were elicited by depolarizations near the foot of the voltage-activation curve (top). Currents are shown before (black) and in the presence of toxin (colored). Normalized tail current voltage-activation relationships are also shown (bottom), where tail current amplitude (I/I_{max}) is plotted against test voltage before (black) and in the presence of toxins (colored). n = 3–5; error bars are s.e.m.

[12]. Thus, ProTx-I inhibits channel opening because it interacts with the paddle motif in domain II and IV, whereas SGTx1 hinders inactivation because it interacts exclusively with the paddle motif in domain IV. Although the domains targeted by Magi5 have yet to be identified, a simple hypothesis would be that the toxin interacts with paddle motifs in domains I, II or III to stabilize at least one of the voltage sensors in an activated state. The competition observed between Magi5 and CssIV [61] (β-scorpion toxin known to interact with the paddle motif in domain II [45]) suggests that Magi5 may interact with the voltage sensor in domain II to facilitate opening.

Toxin–lipid interactions

In the classical view, toxins that affect the gating process of voltage-activated ion channels are thought to interact with voltage sensors through direct protein–protein interactions [22,25,45,50]. However, X-ray structures of Kv channels predict that these voltage sensors are extensively exposed to surrounding lipids if embedded in a membrane [55–57] and functional studies demonstrate that the composition of the lipid membrane affects how Kv channels open and close in response to voltage changes [62–65]. Motivated by these new ideas about voltage sensors, several research groups explored the possibility that tarantula toxins interact with voltage sensors in Kv channels by partitioning into the membrane and binding to paddle motifs at the protein–lipid interface. The amphipatic character observed in the structures of many of these tarantula toxins [39,42–44] is consistent with the notion that membrane partitioning may be required for the toxin to reach the channel. VSTx1, hanatoxin and SGTx1 can partition into model membranes [24,42,66,67] and, in the case of SGTx1, partitioning was observed under physiologically relevant conditions [67]. In addition, modification of native lipid membranes alters the apparent affinity of tarantula toxins, suggesting that these toxins interact with Kv channels within the membrane [67,68]. To explore the possibility that the inhibitory effects of tarantula toxins might result from indirect membrane perturbations without the toxin interacting with the Kv channel, the effects of the D- and L-enantiomers of SGTx1 were compared [67]. The interaction of the two enantiomers with membranes was indistinguishable, but the D-enantiomer was pharmacologically inactive, indicating that the toxin-membrane interaction is not sufficient to inhibit Kv channels.

Although these studies examining membrane interactions focused on toxins that interact with voltage sensors in Kv channels, it was subsequently discovered that hanatoxin and SGTx1 also interact with paddle motifs in Nav channels, implying that partitioning is also involved in toxins interacting with Nav channels. ProTx-II can partition into membranes [32], and the interaction of ProTx-I with the paddle motif from domain IV of Nav1.4 is sensitive to lipid modification [68], consistent with the involvement of membrane partitioning. In contrast, studies with HwTx-IV failed to detect partitioning of the toxin into negatively charged or neutral phospholipid bilayers [41]. However, this does not preclude the involvement of membrane partitioning of the toxin in channel inhibition because the method used is not sufficiently sensitive to detect weak partitioning. For example, Milescu and colleagues showed that SGTx1 partitions into native cell membranes with a mole-fraction partitioning coefficient as low as $\sim 10^3$, an interaction that is barely detectable yet sufficient for the toxin to access its channel interaction site within the membrane [67].

One of the more intriguing discoveries with tarantula toxins is that they can detect the intimate interaction between a specific membrane lipid and voltage sensors in Kv channels and Nav channels [68]. The apparent affinity of tarantula toxins increases after conversion of the membrane lipid sphingomyelin to ceramide-1-phosphate, and the magnitude of the effects varies for different toxins and paddle motifs. The effect of lipid modification differs greatly for mutations in the paddle motif. This suggests that the lipid interacts in a specific fashion with this structural motif, and that the toxin receptor in voltage-activated ion channels is a tri-molecular complex consisting of the voltage sensor, the toxin, and the surrounding membrane lipids. These results also imply that the toxin pharmacology of the ion channel is not determined by the protein alone, but by the lipids in the surrounding membrane and how they interact with the channel protein. For this reason, an exciting goal will be to explore if the membrane properties vary in different cell types or pathophysiological conditions, thereby influencing the gating properties and pharmacological sensitivities of their voltage-activated ion channels.

Promiscuity of voltage-sensor toxins

A unique feature of voltage-sensor toxins is that they can interact with different families of voltage-activated ion channels. For example, hanatoxin was initially isolated during a search for new inhibitors of Kv2.1 [69]. In addition to inhibiting other subtypes of Kv channels such as Kv4.2, hanatoxin can also interact with certain subtypes of voltage-activated calcium (Cav) channels and Nav channels [12,70]. Similar promiscuous activity has been observed for ProTx-I, ProTx-II and SGTx1 [12,29,71,72]. Screening spider toxins for activity on different families of ion channels is not common practice, so other toxins might also be promiscuous. In this respect, it would be interesting to see if Nav channels are affected by closely related peptides that interact with entirely different ion channels (e.g. GsMTx-4 [73] and VaTx1-3 [74]).

The widespread targeting of paddle motifs by animal toxins emphasizes the pharmacological importance of this part of the voltage sensor. However, their amino-acid sequence homology is low, suggesting that toxins probably recognize the conserved three-dimensional structure of the paddle as opposed to a specific sequence of residues [12,21,70]. In addition, the prevalent amphipatic structure of voltage-sensor toxins suggests that toxin–lipid interactions constitute a major part of the interaction mechanism, and may contribute to the promiscuous character of these toxins. We are only beginning to explore the role of membrane lipids in forming the toxin receptor, so their function in toxin promiscuity is an aspect that warrants future study.

Future applications

Although spider toxins have been valuable tools to probe the structure and functional mechanisms of Nav channels,

opportunities also exist for using these toxins therapeutically . Many people experience moderate-to-severe chronic pain that affects their quality-of-life and ability to work. Recent reports concerning the key roles of Nav1.7, Nav1.8 and Nav1.9 in pain perception offers exciting prospects for the discovery of new approaches to analgesia [75]. ProTx-I and ProTx-II were purified on the basis of their ability to reversibly inhibit the activation of Nav1.8 [29]. Unfortunately, these toxins are promiscuous and inhibit other voltage-activated ion channels. However, at the time, these toxins were the first inhibitors of Nav1.8 and provided an important incentive to continue the exploration of spider venoms. Interestingly, Schmalhofer and colleagues subsequently showed that ProTx-II has the highest affinity for Nav1.7, thereby blocking propagation of action potentials in nociceptors [31]. Recently, three more gating-modifier toxins (CcoTx1, CcoTx2 and PaurTx3) were isolated [40]. All of them inhibit Nav1.7 and Nav1.8, albeit with low affinity. Although peptide degradation may limit the use of these toxins *in vivo* in cases warranting local application of anesthetics, they can be valuable tools in proof-of-concept experiments in pain models that require the development of subtype-selective Nav channel-modulators.

Nav1.5 is known to be involved in cardiovascular function, as demonstrated by the catastrophic effects that mutations can cause [4,76]. However, no drug lead has emerged from toxin research for direct application in cardiovascular diseases. Nevertheless, several tarantula toxins have been shown to possess a certain degree of selectivity for this subtype of Nav channel. For example, the recently discovered CcoTx3 was tested on most Nav channel subtypes [40,77] (except Nav1.9) and shown to preferentially inhibit the activation of Nav1.5 (albeit with a weak affinity). Also, JzTx-I [78] and –III [79] modulate cardiac Na^+ currents, presumably by targeting Nav1.5. However, like many other toxins, JzTX-I and -III were not tested on all Nav channel subtypes.

One very important contribution of Nav channel toxins is that they can be used to identify and characterize important relationships between domain-specific interactions and the effect of certain molecules on Nav channel gating. It was recently shown that toxins which slow inactivation of Nav channels bind only to domain IV, whereas toxins that influence opening can do so by interacting with the paddle motifs in domains I–III [12]. Influencing opening of the channel can therefore be accomplished by designing molecules that interact with one or more of the first three voltage sensors in Nav channels, whereas influencing inactivation requires a molecule that interacts only with the domain IV voltage sensor. The influence of domain-specific interactions has important implications for designing drugs to reshape the activity of Nav channels. Diseases associated with accelerated inactivation of Nav channels, such as certain congenital heart disorders, could be managed by drugs that selectively target the domain IV paddle [80]. In contrast, the abnormal opening of Nav channels (as seen in epilepsy disorders) could be controlled with drugs targeting any paddle within the first three voltage sensors [81].

Challenges remain between the initial drug discovery phase and the clinical use of spider toxins because their inherent biochemical instability limits oral availability. Nevertheless, synthetic methods such as cyclization, minimization, and use of diselenide or fluorous bridges have been proposed for stabilizing and locking the conformation of small peptides [82,83], which may expand the use of these toxins as therapeutic drugs .

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