Modulation of TRP Ion Channels by Venomous Toxins

Jan Siemens and Christina Hanack

Contents

Abstract

Venoms are evolutionarily fine-tuned mixtures of small molecules, peptides, and proteins—referred to as toxins—that have evolved to specifically modulate and interfere with the function of diverse molecular targets within the envenomated animal. Many of the identified toxin targets are membrane receptors and ion channels. Due to their high specificity, toxins have emerged as an invaluable tool set for the molecular characterization of ion channels, and a selected group of toxins even have been developed into therapeutics. More recently, TRP ion channels have been included as targets for venomous toxins. In particular, a number of apparently unrelated peptide toxins target the capsaicin receptor TRPV1 to produce inflammatory pain. These toxins have turned out to be invaluable for structural and functional characterizations of the capsaicin

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B. Nilius and V. Flockerzi (eds.), *Mammalian Transient Receptor Potential (TRP) Cation Channels*, Handbook of Experimental Pharmacology 223, DOI 10.1007/978-3-319-05161-1_17,

receptor. If toxins will serve similar roles for other TRP ion channels, only future will tell.

Keywords
Toxin • Vanillotoxin • DkTx • Capsaicin receptor • TRPV1 • TRP ion channel

1 Introduction: Venom Biology

In the animal kingdom, venoms are chiefly used either as part of a predatory strategy to paralyze, capture, and kill prey or as a defense mechanism to ward of predators and/or competitors (Evans and Schmidt 1990).

Additional functions have been proposed, and some insect venom ingredients may serve chemical communication (Speed et al. 2012).

Venoms from spiders, snakes, cone snails, fish, and scorpions are complex cocktails of salts, nucleotides (e.g., ATP), free amino acids, neurotransmitters, polyamines, peptides and proteins. From a pharmacological viewpoint, the latter two toxin groups, peptides, and proteins have attracted the most interest and have been invaluable to dissect and probe the biology of their targeted receptors. A few selected toxins have also inspired drug development (described in the following sections), and there is likely more to be expected in the future.

The astounding specificity, selectivity, and potency of many of the identified toxins for their molecular targets are owed to sophisticated adaptive mechanisms of diversification and expansion of selected toxins and toxin families (Blumenthal and Seibert 2003; Han et al. 2008; Lynch et al. 2006; Terlau and Olivera 2004; Lewis and Garcia 2003; Phui Yee et al. 2004; Twede et al. 2009).

The toxin profiles of different venoms are highly diverse. Diversity is not only found when comparing venoms of different species but a great deal of variability can also be observed when comparing individuals of a given species (Speed et al. 2012). The utility of such variability within a given species is not entirely clear and is not understood if this form of variability represents "ecological noise" or has adaptive evolutionary significance, potentially reflecting adaptation to different habitats.

Despite peptide toxin variability and complexity, there are common principles dictating the nature of structural building blocks and scaffolds. One structural motif, which is shared by many spider, scorpion and cone snail toxins, is the so-called inhibitor cysteine knot (ICK) fold of peptide toxins that are typically comprised of a total of 25–50 residues (Daly and Craik 2011). A hallmark of ICK toxins are the presence of 6 cysteine residues that form intramolecular disulfide bridges, constraining the toxin into a knot-like structure. The ICK fold is among the most abundant toxin motifs, and ICK toxins are estimated to account for 10^5–10^6 unique toxin sequences (Craik et al. 2001; Zhu et al. 2003). Thus, the ICK motif can be considered an "evolutionary vetted" scaffold that provides stability and

protection against proteolytic cleavage and reducing environments when injected into another animal. Additionally, the ICK fold allows for high variability in the inter-cysteine loops that govern target receptor-specific interaction sites (Craik et al. 2001).

Spacing of cysteine residues appears to be important for productive folding, and therefore the number of naturally occurring cysteine patterns found in toxins is limited. The loops in between cysteine residues comprise regions of highly variable amino acid composition, which confer specificity to discrete target ion channel and receptor types. Given the relatively conserved nature of cysteine-residue position, it is suggested that they share a common evolutionary origin (Conticello et al. 2001). This raises the question of how the hypervariable regions in between cysteine residues are generated. A few studies, mainly carried out on fish-hunting cone snails, the so-called conotoxins, have been conducted. These studies suggest that a highly mutagenic molecular replication mechanism is at play, similar to the DNA-Polymerase V in bacterial stress-response mutagenesis (Conticello et al. 2000). At the same time macromolecules binding to cysteine-encoding codon triplets have been postulated to protect and conserve cysteine patterns of the toxin protein product (Conticello et al. 2000; Duda and Palumbi 1999; Olivera et al. 1999). While such a scenario is attractive and could explain the high diversity of cysteine-knot toxins, components of such a mutagenic replication machinery or molecules binding to specific DNA or RNA triplets have not been identified from venom ducts. Future studies are required to unveil and characterize this putative mechanism and reveal whether it can be exploited to generate pharmacologically relevant toxin libraries in vitro.

It has been proposed that the ICK motif has emerged from a simpler, ancestral toxin fold, encompassing only two disulfide bonds, the so-called disulfide-directed β-hairpin (DDH) (Escoubas and Rash 2004; Smith et al. 2011; Wang et al. 2000).

While the DDH motif is found in many proteins and in most phyla, direct evidence for molecular evolution from DDH to ICK motif was lacking until recently, when a scorpion toxin was identified and shown to adopt to this previously hypothetical DDH fold (Smith et al. 2011). By comparisons of ICK structures with that of the newly identified DDH toxin, it has been found that the two central disulfide bridges of ICK toxins nicely align with the DDH disulfides, suggesting that the "outer" solvent exposed additional disulfide, that closes the ring of the ICK motif, has emerged later during evolution (Fig. 1).

While many venoms can cause inflammatory pain as a consequence of severe tissue damage, some venoms can produce a robust perception of pain without eliciting appreciable trauma or damage. As illustrated below, venoms of the latter category have been found to directly engage and hijack ion channels and receptors of nociceptive sensory neurons, among them TRP ion channels. Likely, this serves to deter and avert predators and competitors by inflicting a painful and memorable experience. This parallels the mechanisms adopted by numerous plant species to deter predatory mammals through the production of chemical irritants (such as capsaicin or isothiocyanates) that also target TRP channels on sensory neurons of the pain pathway (Basbaum et al. 2009).

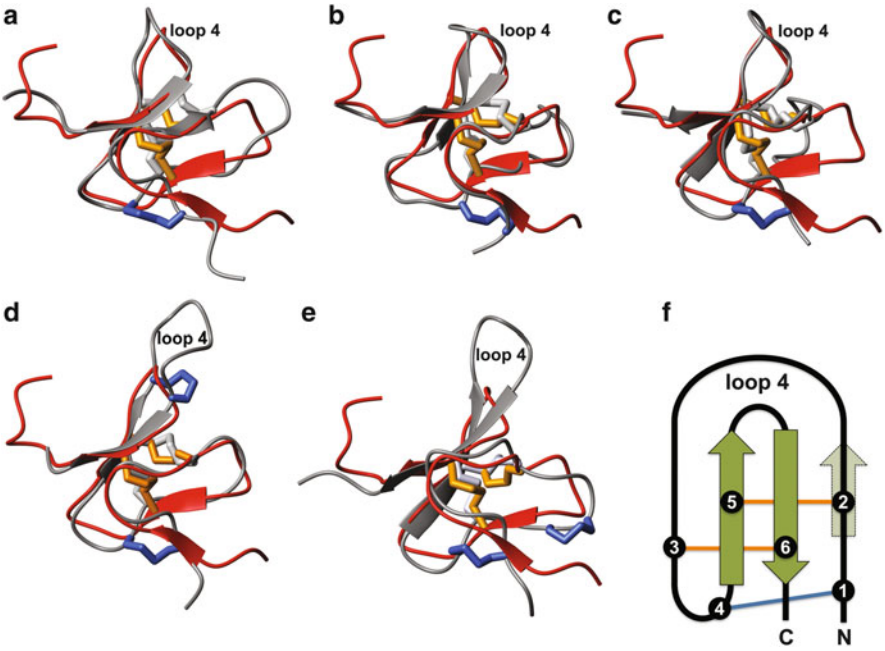

Fig. 1 Overlay of the newly identified ancestral DDH toxin (U1-LITX-Lw1a, *red*) with homologous ICK toxins (*gray*). Homologous spider ICK toxins include (**a**) guangxitoxin, (**b**) GsMTX-4, (**c**) Hainantoxin-I, (**d**) Purotoxin, and (**e**) κ-hexatoxin-Hv1c. The two central disulfides of the ICK toxins shown in *gray* tubes overlap with the two disulfides of DDH (*gold tubes*). Additional ICK disulfides are shown in *blue*. (**f**) Graphical representation of the DDH and ICK motifs, with the disulfides and β-sheet of the DDH motif shown in *orange* and *green*, respectively. The third disulfide necessary for the formation of the ICK motif is shown in *blue*. (Adopted with permission from Smith JJ. et al. 2011, *PNAS*, Vol. 108, 26, 10478–10483.)

2 Toxins of Venomous Organisms in Ion Channel Research and Medical Therapy

A lot of the knowledge on ion channel structure and function (ligand or voltage-gated) was elucidated using specific purified or synthesized peptide toxins (Catterall 1986; Norton and Olivera 2006; Terlau and Olivera 2004; Tsetlin 1999).

The Tarantula toxin, Hanatoxin, for instance, helped to pave the way for structure-function analysis of potassium and sodium ion channels. Other known examples include tetrodotoxin (TTX) from tetraodon pufferfish and saxitoxin (STX) from shellfish (Llewellyn 2006) for blocking sodium channels. Both toxins have revealed valuable information about the way neurons communicate with each other. α-bungarotoxin and other α-neurotoxins from *Elapidae* and *Hydrophidae* snakes were used to provide the first identification of nAChRs (Hucho et al. 1996; Lena and Changeux 1998), and α-bungarotoxin is still used as the most reliable

pharmacological tool to study neuromuscular blockage. Furthermore, α- and β-neurotoxins from scorpions have been used to modulate sodium channels by delaying inactivation (α), or shifting the membrane potential dependence (β) (Bosmans and Tytgat 2007; Zuo and Ji 2004). The high degree of specificity with which venom peptides bind to different ion channel families as well as to other membrane receptors (including voltage-gated sodium (Nav), calcium (Cav), and potassium (Kv) ion channels and ligand-gated receptors such as nicotinic acetylcholine receptors (nAChRs), *N*-methyl-D-aspartate (NMDA), and G-protein coupled receptors (GPCRs)) make them ideal tools to study biophysical properties of target receptors as well as their physiological functions in vivo. Additionally, their high degree of specificity makes them suitable for manipulating the activity of selective cell subtypes. These properties (specificity/selectivity and high biological activity) that make toxins effective venoms are also what make them so valuable for medical applications. Many venom toxins target the same molecules that need to be controlled during diseases. A specific therapeutic area in which peptide toxins have already proven their potential includes, for instance, the treatment of chronic pain in humans by ω-conotoxin MVIIA (commercialized as Prialt) (Miljanich 2004; Staats et al. 2004; Zamponi et al. 2009). Snail venom peptides called conantokins are being tested with some success against epileptic seizures (Jimenez et al. 2002). Both, conotoxins and conantokins, are evaluated in studies of Alzheimer's and Parkinson's diseases, depression, and nicotine addiction (Moriguchi et al. 2012; Ragnarsson et al. 2002). In 2005, a venom component of Gila monster, called Exenatide (Byetta), was approved by the US Food and Drug Administration for the management of type 2 diabetes mellitus (Bond 2006). This peptide stimulates the secretion of insulin in the presence of elevated blood glucose levels. The sea anemone holds great potential for treating autoimmune diseases such as multiple sclerosis, rheumatoid arthritis, psoriasis, and lupus (Chi et al. 2012). For instance, Stichodactyla toxin (ShK) blocks Kv1.1 and Kv1.3 channels and is currently in phase 1 human trials for the treatment of autoimmune diseases. Eastern green mambas possess toxins that impair blood circulation and could be life saving to heart patients; Cenderitide, a fusion of a key peptide of the venom with a peptide from cells of human blood vessels, is intended to lower blood pressure and reduce fibrosis in a failing heart (Martin et al. 2012; von Lueder et al. 2013). Interestingly, ASIC channels are important targets by various venomous toxins. These include the sea anemone toxin, APETx2, that diminishes postoperative pain (Diochot et al. 2004), as well as psalmotoxin 1 (PcTx1) of the Trinidad chevron tarantula and Black mamba toxin peptides (Mambalgins) targeting ASIC channels with great potential to be effective new analgesics in the treatment of chronic pain (Diochot et al. 2012; Mazzuca et al. 2007). Moreover, a drug based on an anticoagulant toxin in the saliva of the vampire bat is now in clinical trials to test its potential to dissolve blood clots of stroke patients. It was found that the thrombolytic activity of the toxin exceeds that of conventional treatments, extending the time window for treatment from currently 4 to potentially 9 h (Liberatore et al. 2003; Medcalf 2012; Reddrop et al. 2005).

3 Toxins: Mode of Action

The mode of action of toxins will be exemplified on Kv channels, which have been most extensively studied. A number of different mechanisms have evolved for toxin-mediated modulation of ion channel activity. Among the best studied examples are two major groups: The "pore-pluggers" and the "voltage-sensor modulators."

"Pore-pluggers" bind to the outer vestibule of the ion conduction pore and physically block the flow of ions. Well-characterized examples are the scorpion toxins, charybodotoxin and agitoxin (Miller 1995), which block potassium channels and were used to identify and probe their pore region (MacKinnon and Miller 1989). The pore domain determines K^+ ion selectivity and contains the region that pore blocking toxins target. In the case of charybdotoxin, a Lys residue on the active surface of the toxin is "plugged" into the pore of the channel and interacts with potassium ions bound within the selectivity filter (Anderson et al. 1988; Banerjee et al. 2013; MacKinnon and Miller 1988; Park and Miller 1992; Rodriguez de la Vega et al. 2003).

The voltage-sensing domain is a second region of ion channels that is widely targeted by toxins. The four external arginine (or lysine) residues in S4 are positively charged and carry most of the gating charge (Aggarwal and MacKinnon 1996; Seoh et al. 1996). Binding to this region induces conformational changes during gating and thereby influences the gating mechanism by altering the stability of the closed, open, or inactivated states. Toxins targeting the voltage sensing domains of Nav channels can block or facilitate channel opening (Bosmans and Swartz 2010). Extensive studies on the voltage sensor were carried out in Kv channels. Hanatoxin, a 35-aa three-disulfide bond peptide, is the best-studied tarantula toxin that targets the voltage sensor and inhibits opening of the Kv channel by stabilizing the resting conformation of the voltage sensor (Phillips et al. 2005; Swartz and MacKinnon 1997a, b). As illustrated in Fig. 2 the region where hanatoxin binds is called the voltage sensor paddle motif (Jiang et al. 2003b), a helix-turn-helix motif composed of the C-terminal portion of S3, (S3b), and the S4 helix (Alabi et al. 2007; Jiang et al. 2003a; Ruta et al. 2005; Swartz 2008). This is a mobile region in the voltage-sensing domain that moves in response to changes in membrane voltage (Alabi et al. 2007; Jiang et al. 2003a, b). It was shown that paddle motifs are modular components that can be swapped between ion channels with a voltage-sensing domain without losing their functional properties (Alabi et al. 2007; Bosmans et al. 2008). The paddle motif is an important pharmacological target in ion channels because various tarantula toxins were shown to interact with this region (Bosmans et al. 2008; Milescu et al. 2009; Phillips et al. 2005; Lee et al. 2004). In addition, it was postulated that toxins like hanatoxin partition into the lipid membrane and bind to the paddle motif from within the bilayer (Lee and MacKinnon 2004; Milescu et al. 2007, 2009) (See also Fig. 2). This suggests that the effect of toxins and their interaction with the ion channel is not determined by the protein alone but by the lipids in the surrounding membrane.

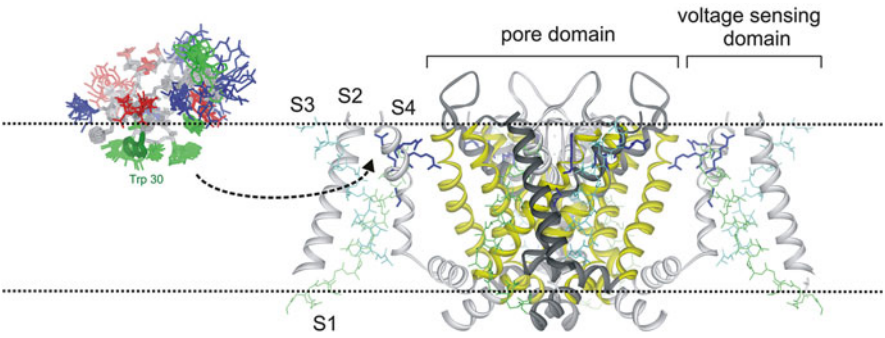

Fig. 2 Illustration of hanatoxin binding to the voltage sensor of a Kv channel. Hanatoxin, a 35-aa three-disulfide bond peptide, is among the best-studied tarantula toxin that targets the voltage sensor domain and inhibits opening of the Kv channel by stabilizing the resting conformation of the ion channel. The region where hanatoxin binds is the so-called voltage sensor paddle motif, a helix-turn-helix motif composed of the C-terminal portion of S3, (S3b) and the S4 helix. This mobile region in the voltage-sensing domain moves in response to changes in membrane voltage. It has been suggested that Hanatoxin partitions into the lipid membrane and binds to the paddle motif from within the bilayer. (Adopted with permission from Swartz 2007, Toxicon, Vol. 49, Issue 2, 213-230)

4 Toxins Modulating TRPV1 Activity

4.1 Vanillotoxins

TRPV1 is predominantly expressed in primary afferent sensory fibers that innervate the skin and viscera. Originally cloned as the receptor for capsaicin, the pungent ingredient in red chili peppers (Caterina et al. 1997), TRPV1 has subsequently emerged as a prototypical molecular nociceptor, participating in the detection of a wide range of noxious stimuli such as low pH, high temperature, and inflammatory signaling molecules (Basbaum et al. 2009). Biophysical properties as well as physiological functions of TRPV1 are described in detail in a previous chapter of this book.

Pain serves as an important warning system signaling tissue damage and distress triggering protective reflexes that allow the organism to respond to and escape from potentially dangerous environments.

Similar to selected plant species, venoms of some animals inflict pain by directly activating primary afferent sensory fibers, presumably hijacking the pain system, to deter predators and competitors by causing a memorable discomfort and distress.

Since TRPV1 and its related ion channels TRPA1 and TRPM8 are central to the pain pathway, a screen was conducted to test whether venoms contain toxins specifically activating these receptors thus explaining a pain-producing effect of bites and stings by some venomous species. Out of 22 spider and scorpion venoms, two distantly related tarantula species, *Psalmopoeus cambdridgei* and

Ornithoctonus huwena (the latter also known as "Earth Tiger"), robustly and specifically activated TRPV1 (Siemens et al. 2006). Reiterative reversed-phase chromatography of *Psalmopoeus cambdridgei* venom yielded three related peptide toxins each of which individually promoted TRPV1 activation with different efficacies in calcium imaging experiments and electrophysiological recordings of overexpressed or native vanilloid receptor TRPV1. Accordingly, the peptide toxins were named vanillotoxins (VaTx 1–3). Edman peptide sequencing combined with mass spectrometry revealed that vanillotoxins consist of 34–35 amino acids that belong to the extended family of inhibitory cysteine knot (ICK) peptides. Vatxs were also confirmed to elicit nocifensive behavior in a TRPV1-dependent fashion and to be present in sufficiently high enough concentrations in the crude venom (100–1,000 Fold their EC50 values), suggesting that a TRPV1-mediated pain response can be triggered in envenomated animals in the wild.

ICK toxins are widely recognized as inhibitors of cationic channels and transmembrane receptors, exemplified by the detailed analysis of Hanatoxin's (Hatx1 and 2) interaction with voltage-gated potassium (Kv-) channels (Swartz 2007). Vanillotoxins were among the first ICKs identified that act as positive modulators of a cationic channel rather than serving as inhibitors.

To test whether any of the VaTxs additionally exhibit a "classic" inhibitory function, they were tested on a set of Kv channels. Indeed, it was found that Vatx1 is equally potent as a TRPV1 activator and as a Kv2.1 inhibitor (Fig. 3). Vatx 2 and 3, that are more potent TRPV1 activators than VaTx1, had only minor or no appreciable activity on any of the tested Kv channels (Siemens et al. 2006). The diversification of vanillotoxins serves as a rare illustration how one of the most commonly found scaffolds in venom toxins, the ICK motif, can be adapted to novel functions. As described above, the Cysteine knot is a very robust, yet at the same time highly versatile structural scaffold, serving many functions that are acquired by gene duplication and rapid diversification (Conticello et al. 2001; Sollod et al. 2005; Kordis and Gubensek 2000). Here, Vatx 1-3 exemplify apparent evolutionary transition states with graded selectivity for Kv2.1 and TRPV1 ion channels (Bohlen and Julius 2012). This functional diversity is also reflected in sequence differences: Among the 3 vanillotoxins, promiscuous Vatx1 is most similar to Kv inhibitor toxins from related tarantulas (such as the Heteroscodra maculate toxin HmTx1) while Vatx2 and Vatx3 are progressively dissimilar to Vatx1 (and for that matter to HmTx1) and at the same time more specific and potent activators for TRPV1.

Both channel types, Kv and TRP ion channels, are believed to be structurally related and share similar membrane topology, tetrameric organization, and to a limited degree also voltage sensitivity. Given this similarity, one would intuitively speculate that VaTx1 is likely to bind to a similar region within Kv2.1 and TRPV1 ion channel polypetides. Surprisingly, directed mutagenesis as well as use of chimeric TRPV1 channels encompassing domains of toxin-insensitive frog TRPV1 and toxin-sensitive rat TRPV1 showed that this is not the case (Bohlen et al. 2010). While VaTx1 inhibits Kv channels by binding to the voltage sensor domain (the third and fourth transmembrane helices) (Fig. 2) much like other

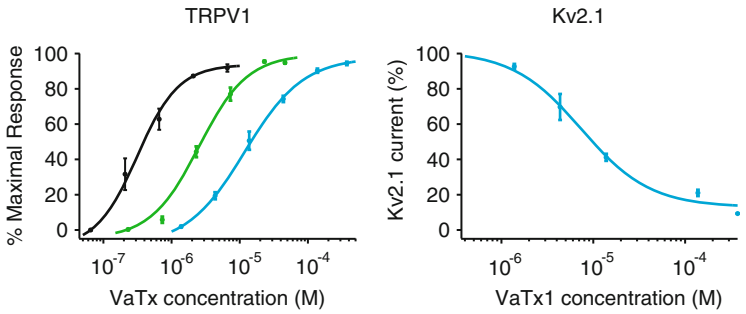

Fig. 3 Vatx1 can act both, as an activator of TRPV1 and an inhibitor of Kv2.1. *Left*: dose–response analysis by whole-cell voltage-clamp recording of TRPV1-expressing oocytes (180 mV) revealed EC50 values of 11.9 ± 1.4, 2.53 ± 0.02 and 0.32 ± 0.09 μM for VaTx1 (*blue*), VaTx2 (*green*), and VaTx3 (*black*), respectively. *Right*: toxin-mediated inhibition of Kv2.1 (at 0 mV) was assessed in oocytes. The full dose–response is shown for VaTx1 ($IC_{50} = 7.4 \pm 1.9$ μM). Error bars represent s.e.m.; $n > 3$ trials for each toxin concentration. (Adopted with permission from Siemens, J. et al. 2006, *Nature*, Vol 444)

extensively studied ICK toxins (Catterall et al. 2007; Swartz 2007), the toxin instead recognizes the pore domain (the fifth and sixth transmembrane helices) within TRPV1 protein (Bohlen et al. 2010) (Fig. 4). Opposite to Vatx1, it appears that VaTx2 and 3 have evolutionary refined their binding specificity in favor of a TRPV1 interaction.

There are several examples of toxins that have acquired new functionalities over time and others appear to be in the middle of such a transitioning process having adopted bifunctionality, such as huwentoxin-XI, which has acquired both trypsin and Kv channels' inhibitory activity (Fry et al. 2009; Yuan et al. 2008).

As such, the different vanillotoxins could be considered evolutionary intermediates transitioning between paralyzing (Kv channel inhibiting) and pain-inducing (TRPV1 activating) toxin functionalities.

4.2 Double-Knot Toxin

Although the chromatographic profile of the active ingredient in *Ornithoctonus huwena* venom suggested that it was also proteinacious in nature, with a mass of 8,522 Da it appeared unusually large for an ICK peptide.

Due to paucity of material and its rather large size, a different strategy was employed to disclose its nature. A partial sequence was obtained by de novo peptide sequencing. The peptide sequence information was used to design degenerate primers for subsequent PCR cloning of the full-length cDNA encoding the mature toxin from venom gland tissue (Bohlen et al. 2010). The *O. huwena* toxin had no apparent homology to any of the three vanillotoxins, suggesting that the TRPV1-activating toxins of the two tarantula species had independently evolved through convergent evolution. Closer analysis revealed that, most strikingly, the toxin

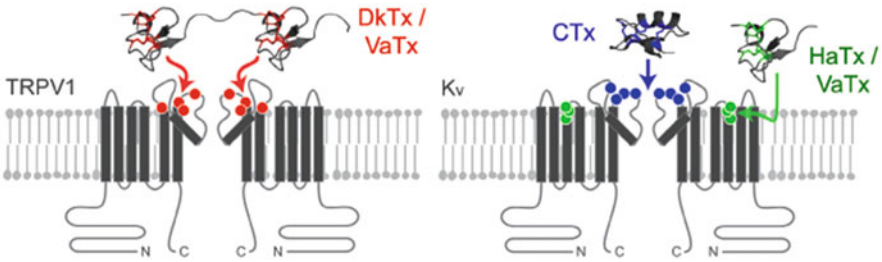

Fig. 4 Model of Toxin binding to TRPV1 and Kv channels. *Gray* bars represent transmembrane helices, and *red* dots highlights residues that are crucial for double-knot toxin (DkTx) activation. In the simplest scenario, the two knots of DkTx bind to two equivalent sites on multiple subunits of the same channel. Kv channels likely possess the same overall transmembrane topology as TRPV1 but interact with ICK toxins in different ways. For example, charybdotoxin (CTx) binds within the ion permeation path to block ion flux, and voltage-modulator toxins, such as hanatoxin (HaTx), target the voltage sensor to modify gating properties (*blue* and *green* dots represent mutations that attenuate CTx and HaTx inhibition, respectively). The single-knot vanillotoxins (VaTx) also appear to target the S3–S4 helices of Kv channels, but they activate TRPV1 through the pore region. (Adopted with permission from Bohlen CJ. et al. 2010, *Cell*, Vol. 141, p. 834–845)

consisted of tandem repeats of two individual ICK motifs and was therefore named "double knot toxin" (DkTx, Fig. 4). Presumably, DkTx has evolved from a gene duplication event that conjoined the two individual ICK motifs into a single open reading frame.

This unusual structural arrangement is paralleled by nearly irreversible activation of its target receptor TRPV1. A series of experiments led to the conclusion that the two covalently connected ICK lobes have bivalent properties, resulting in exceptionally high avidity for its multimeric target TRPV1, similar to antibody-like binding properties. Indeed, robust and long-lasting activation of TRPV1 correlated with specific, nearly irreversible direct physical interaction of DkTx with the ion channel, as deduced from binding studies (Bohlen et al. 2010).

Multimerizing ligands as a means to increase their effectiveness is a well studied and exploited phenomenon in pharmaceutical chemistry. There are numerous examples were multivalent strategies have proven extremely useful and result in enhanced kinetic parameters and drastically increased affinities of pharmacophores (Bohlen and Julius 2012). The mode of action of such multivalent ligands can be categorized into three different modes: polyvalency (targeting multiple receptors distributed over a larger surface), hetero-multivalency (targeting discrete epitopes of a given receptor), and homo-multivalency (targeting identical binding sites on different subunits of a multimeric complex). Initial estimations accounting for DkTx linker length estimations accounting for DkTx linker length as well as the presumed dimensions of homo tetrameric TRPV1 channels suggest that homo-multivalency is the likeliest scenario for a TRPV1–DkTx mode of interaction. Theses findings are corroborated by subsequent structural analysis of a DkTx-TRPV1 protein complex (Cao et al. 2013). Single channel recordings of TRPV1 in the presence of DkTx are in agreement with this interpretation: irreversible

activity of DkTx is not only observed in macroscopic currents deduced from whole-cell patch-clamp recordings but have been also recapitulated in single channel recordings of TRPV1 (Bohlen et al. 2010).

While multivalency is a widespread phenomenon in nature as well as pharmaceutical chemistry, it has thus far been rarely observed for toxins. Few reported examples include sarafotoxin as well as a newly identified tandem ICK toxin from the yellow sac spider as well as a few selected other examples (Ducancel et al. 1993; Vassilevski et al. 2010). Given the widespread presence of longer peptide toxins (>6,000 Da) in scorpion, cone snail, and spider venom (Escoubas and Rash 2004), it is likely that more tandem repeat ICK toxins will be discovered in the future.

Other non-covalently linked multimeric toxins have been identified that also have multivalent properties, such as the highly potent two-component toxin from a coral snake that activates acid-sensing ion channels (ASICs) (Bohlen et al. 2011). Interestingly, ASIC activation by MitTx toxin complex also induces pain and inflammation, again pointing to an adaptation selecting for exploitation of the pain pathway of a variety of venomous animals inhabiting different environmental niches.

While the TRPV1 activator DkTx is not the only multivalent toxin, it represents thus far the clearest case that bivalency can tremendously increase potency as well as duration of activity compared to individual, monovalent vanillotoxins. DkTx can be produced in good yields, and researchers are not required to rely on miniature amounts that can be harvested from spiders (Bae et al. 2012). These properties of DkTx not only aided in probing biophysical properties and physiological functions of TRPV1, it also helped to obtain the first high resolution structures in the open conformation of the capsaicin receptor, or for that matter, of any TRP ion channel (Cao et al. 2013; see also a previous chapter in this book, highlighting TRPV1 protein structures).

Since natural toxins target a number of medically relevant receptors with high specificity and have been invaluable tools in basic research that have additionally also entered the therapeutic stage, a combinatorial approach to generate multivalent toxins may greatly improve toxin effectiveness and should be explored in the future.

5 Additional TRPV1-Mediated Toxin Effects

A number of venoms have been found to cause inflammation and pain, either locally but sometimes also more widespread and distal to the site of the bite or sting. Certainly, not all of these effects can be attributed to TRPV1 activation, and other molecular toxin targets of the somatosensory system have been identified that can mediate painful responses (Bohlen and Julius 2012). Nevertheless, some additional toxins have been found to target TRPV1, either directly or indirectly. Among them are a group of polycyclic ether toxins that are produced by marine dinoflagellates and that are carried by certain reef- and shellfish. Consumption of

such infested tropical fish can lead to ciguatera fish poisoning (CFP) and neurotoxic shellfish poisoning (NSP). Common symptoms of acute intoxication include tingling of lips, hands and feet, unusual temperature perception, and burning mouth syndrome (Cameron and Capra 1993; Heir 2005). Polycyclic ether toxins gambierol and brevetoxin are derived from CFP and NSP, respectively. Cuypers et al. found that both toxins can activate TRPV1 (Cuypers et al. 2007). Both toxins appear to be allosteric modulators of TRPV1 that cannot activate the ion channel on their own (at least not at the concentrations tested) but instead enhance the effect of capsaicin or other TRPV1 agonists. Similar results were found with extracts from jellyfish tentacles that are known to cause a burning pain sensation (Cuypers et al. 2006). While it cannot be ruled out that these toxins have other molecular targets, it is likely that they act directly on TRPV1. Interestingly, similar to Vatx 1, Gambierol not only promoted activation of TRPV1 but also has inhibitory activity on Kv channels (Cuypers et al. 2008). However, the inhibitory mechanism of Gambierol-mediated Kv-channel inhibition appears to be different from ICK toxins such as HaTx or VaTx, and the site of action resides close to the Kv-ion channel pore and not within the voltage-sensor domain (Kopljar et al. 2009). The site of Gambierol's action on TRPV1 has not been determined yet. If Gambierol also constitutes an adaptive transition state of a toxin that acquired a new function, we can only speculate. It may turn out informative to test-related polycyclic ether toxins for their ion channel specificity and to determine structural elements within the polymer that dictate functionality.

Within the pain pathway, TRPV1 cannot only be activated directly by heat or by interactions with agonists such as endogenous produced lipid intermediates and protons, but it is also target of several signaling cascades that activate or sensitize the receptor (Fig. 5). Thus, TRPV1 serves as a molecular integrator (also referred to as polymodal nociceptor) of inflammatory and noxious signals (Julius and Basbaum 2001; Nieto-Posadas et al. 2011; Tominaga and Tominaga 2005). Intriguingly, venomous creatures appear to likewise exploit indirect activation and sensitization mechanisms of TRPV1 to produce a noxious sensation. Bv8, a protein toxin found in skin secretions of the yellow-bellied frog, *Bombina variagata*, is a potent activator of the prokineticin receptor 1 and 2 (PKR 1 and 2). Injection of Bv8 caused a PKR-mediated hyperalgesia that was largely dependent on TRPV1 (Negri et al. 2006; Vellani et al. 2006). Similar toxins have been identified from other reptiles as well as spiders, suggesting that nociceptor sensitization through the manipulation of signaling cascades is more widespread (Fry et al. 2006; Negri et al. 2007; Schweitz et al. 1990; Szeto et al. 2000; Wen et al. 2005) (Fig. 5).

TRPV1 inhibitory activities have also been found in venoms. Partial TRPV1 inhibition has been observed from tropical sea anemone *Heteractis crispa* venom. The polypeptide toxin APHC1 antagonized TRPV1 currents and capsaicin-induced nocifensive responses in animal models (Andreev et al. 2008).

Two non-peptidic toxins, AG489 and AG505, which attenuate TRPV1 activity have been isolated from the funnel web spider, *Agelenopsis aperta*. However, these toxins appear to inhibit a broad range of different receptors and can also lead to

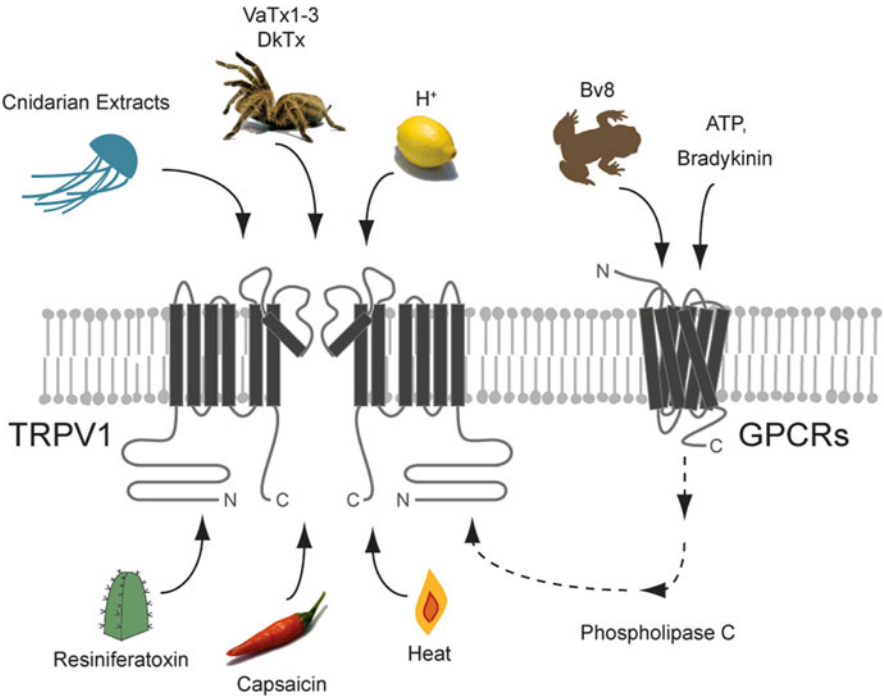

Fig. 5 Toxins targeting TRPV1. TRPV1 detects physical and chemical signals from the environment, including acidic pH and hot temperatures. Plant-derived irritants, cnidarian extracts, and toxins from tarantula venom mimic these harmful stimuli by promoting TRPV1 activation. TRPV1 is sensitized by phospoholipase C activation triggered by inflammatory signaling molecules such as bradykinin or ATP that are released downstream from venom lipases, proteases, and kallikreins. Other toxins, such as Bv8 from frog skin, produce TRPV1 sensitization through direct activation of GPCRs. (Adopted with permission from Bohlen CJ., Toxicon, 2012, Vol. 60, p. 254–264)

TRPV1-independent paralysis of envenomated species (Kitaguchi and Swartz 2005; Skinner et al. 1989).

6 Toxins Affecting Other TRPs

So far, there is only very little information about animal toxins targeting TRP channels other than TRPV1. However, a few other examples exist and the latter two illustrate that toxins indirectly affecting TRP channel function can also help to decipher their physiological roles.

6.1 TRPV6

TRPV6 is expressed in apical membranes of various tissues including kidney, intestine, pancreas, and prostate (Nijenhuis et al. 2003a, b; Zhuang et al. 2002). In the gastrointestinal tract, it is involved in apical entry of calcium ions (Hoenderop et al. 2005; Zhuang et al. 2002). TRPV6 has been implicated in tumor development and progression in a number of cancers (Bodding 2007; Nilius et al. 2007; Santoni et al. 2011). Upregulation of TRPV6 protein levels has been reported in tumors of ovary, breast, colon, and prostate cancer (Wissenbach et al. 2001; Zhuang et al. 2002). This indicates that TRPV6 could be a novel therapeutic target for the treatment of tumors over-expressing the receptor. To elucidate this further, a recent study made use of the toxin Soricidin isolated from the saliva glands of the northern short-tailed shrew (*Blarina brevicauda*) (Peters et al. 2012). Soricidin is a novel peptide that has been shown to inhibit calcium uptake via TRPV6 channels. Furthermore, it was shown that two peptide sequences from the c-terminal domain of soricidin have a high binding affinity for TRPV6 in ovarian cancer. In the study of Bowen et al. (2013), these two peptide sequences were conjugated with a fluorescent dye or an magnetic resonance imaging (MRI) contrast agent which enabled them to monitor the distribution of the peptides in vivo (Bowen et al. 2013). Imaging tumors over-expressing TRPV6 provides opportunities for early detection of ovarian cancer. This is especially important because ovarian cancers are difficult to detect in their early stages, and early detection could be lifesaving.

6.2 TRPA1

TRPA1 is another TRP channel family member that is indirectly modulated by a venomous compound. TRPA1 channels are expressed in nociceptive DRG neurons and are important transducers of pungent or irritating environmental stimuli and thus play a crucial role in nociception. Similar to TRPV1, the receptor has recently been implicated in the development of ciguatoxin-induced symptoms (Vetter et al. 2012). As stated above, Ciguatera fish poisoning is an acute intoxication resulting from the consumption of tropical reef fishes (Lewis 2006). Symptoms of this poisoning also include major peripheral sensory disturbances, including a hypersensitivity to cold (allodynia), which is characterized by intense stabbing and burning pain in response to mild cooling. Of the different ciguatoxin variants that exist, P-CTX (Pacific Ocean CTX) is the most potent and thought to be responsible for the majority of neurological symptoms (Lewis 2001). Interestingly, Vetter et al. now discovered that Ciguatoxin-induced allodynia is due to indirect activation of TRPA1 ion channels. Evidence for this is based on heterologously expressed TRPA1 which could only be activated by P-CTX in the presence of Na (v) channels, not if expressed individually. Importantly, mice lacking TRPA1 showed a reduction of the effect of CTX on C-fibers and on ciguatoxin-induced cold allodynia. Furthermore, functional MRI studies revealed that ciguatoxin-

induced cold allodynia enhanced the BOLD (Blood Oxygenation Level Dependent) signal, an effect that did not occur in TRPA1-knockout mice. As a consequence of ciguatoxin-induced activation of voltage-gated sodium channels, TRPA1 channels are activated at a temperature where they usually would not be activated. Thus, when ciguatoxins are present, a temperature decrease can lead to activation of TRPA1 channels, leading to the inappropriate sensation of painful burning. This may serve as a warning device to alert the body of danger (Voets 2012).

6.3 TRPCs

Bee stings cause persistent pain and swelling around the sting area. The main peptide of bee venom responsible for pain is the toxin melittin. This toxin activates different subpopulations of primary nociceptive neurons. It was recently shown that TRPC channels may represent a possible target by melittin (Ding et al. 2012).

TRPCs are the closest homologs to drosophila TRP channels and are expressed in the central and peripheral nervous system (Chung et al. 2006; Riccio et al. 2002). In the DRG, TRPC1, TRPC3, TRPC4, and TRPC6 are most abundant (Kress et al. 2008). To date, the role of TRPC channels in the somatosensory neurons is not very well established. Ding et al. (2012) recently postulated a possible role for this subgroup of TRP channels in somatosensation. For their study they used the melittin model to induce inflammatory pain (Chen and Lariviere 2010) in rats. Subcutaneous melittin injection causes persistent pain and pronounced thermal and mechanical hyperalgesia (Chen et al. 2006; Li and Chen 2004). Melittin was already shown to indirectly activate TRPV1 (Du et al. 2011). Interestingly, a subpopulation of neurons positive for TRPC1 and TRPC3 does not express TRPV1 but is sensitive to melittin (Ding et al. 2011; Kress et al. 2008), suggesting that TRPV1 is not the only target of this toxin. For this reason, Ding et al. investigated melittin-induced pain behaviors in the presence of the nonselective TRPC antagonist SKF-96365. In fact, administration of this blocker prior or after subcutaneous melittin injection reduced the development of persistent spontaneous pain, suggesting that TRPC channels may be involved in mediating the pathophysiological processes of a bee sting. However, additional experimentation will be needed to fully elucidate the mechanism of melittin-evoked nociception. Due to nonselective properties of SKF-96365, it cannot be ruled out that channels other than TRPCs are at play here.

7 Discussion and Outlook

While it is appealing and intuitive to assume that pain-inflicting toxins provide an adaptive advantage by averting predatory threats, it is difficult to assess their physiological role directly in the wild. As a starting point for addressing this evolutionary aspect, it may be helpful to categorize venomous species into ones that utilize their venom mainly to capture and kill prey and others that employ their

toxin cocktails foremost as a defense mechanism. The assumption would be that venoms used as a defense mechanism may contain pain-inflicting "nocitoxins," while predatory species have less or no nocitoxins since it would not provide an advantage to them. In fact, it may even be disadvantageous to produce a pain response when prey paralysis is the main purpose of the toxin cocktail. In agreement with this notion, toxin combinations have been found in predators that suppress the fight-or-flight response in prey (Olivera et al. 1999).

For some species such as spiders, the venom serves multiple functions and is optimized to encompass both goals of prey capture and chemical defense. In fact, some components of certain insect venoms may even serve less appreciated roles as pheromones and in chemical communication (Speed et al. 2012). But clearly, predatory cone snails chiefly (if not exclusively) use their venom to paralyze and kill prey and thus may have no adaptive need for nocitoxins. In this regard it is noteworthy that a cellular screen similar to that published in Siemens et al. (2006) has also been conducted with venoms of a dozen different cone snail species. In opposite to spider venoms, this screen did not yield any agonist activity on any of the three somatosensory TRP ion channels tested, namely TRPV1, TRPA1, and TRPM8 (David Julius and Jan Siemens—unpublished data). While more comprehensive studies are required to obtain conclusive results for the two categories (prey-capture venoms vs. defensive venoms), these preliminary result argues that in regard of inflicting a painful sensation, there may be adaptive differences.

For the elaboration of this hypothesis, it is also important to consider the perspective of the other party involved, that of the predator or competitor. One particular example illustrates how an adaptive advantage of a pain-inflicting toxin from certain scorpion species, the Bark scorpions, has been negated by a subsequent evolutionary adaption of its predator, the Grasshopper mouse. The venom of Bark scorpions inflicts pain in many mammals such as house mice, rats, and humans by directly activating voltage-gated Na^+ channels. Grasshopper mice express a Na^+ channel variant that is inhibited by the scorpion toxin, thereby blocking action potential propagation and a painful sensation (Rowe et al. 2013). This is an example of the adaptive "arms race" and illustrates the intricate predator—prey relationship, even at the level of somatosensory processing.

It may not be surprising that a number of different venom components target TRPV1, thereby causing a noxious sensation. After all, this is what venoms of many species have evolved for: to deter predators and competitors. Apparently, this can easily be achieved by injecting (through bites and stings) a TRPV1 agonist. ASIC channels are also included as venom targets to produce a noxious sensation (Bohlen et al. 2011) confirming that nocitoxins are not limited to exclusively targeting sensory TRP ion channels. On the other hand, it is somewhat surprising that not more pro-nociceptive toxins have been identified from venomous species that target other receptors relevant to the pain pathway. A number of plant-derived products have been found to activate other sensory receptors of the TRP ion channel family besides TRPV1 (Vriens et al. 2008); however, no additional toxins have been described for these thus far.

Why toxins specific for additional TRPs have not yet been identified, we can only speculate. One trivial explanation is that people have either not searched for such toxins or no robust cellular assays are available for certain TRPs to test toxin activity. Another, more provocative, possibility is that either activation or inhibition of other TRP ion channels does not provide an adaptive advantage to the venom-producing species since it would neither lead to paralysis/death nor a memorable nocifensive response. Thus, toxins affecting other TRP channels may not have been evolutionary selected for.

Yet another possibility is that the gating mechanism of other TRPs may differ from that of TRPV1 and may not be compatible with toxin bioactivity. Most TRP ion channels harbor only relatively small extracellular domains that can be accessed by peptide toxins, which are not able to freely permeate into the cytosol. Binding sites for TRP channel agonists are primarily located intracellularly, which may explain why these receptors are only weakly influenced by extracellular peptides.

In this regard the irritant receptor TRPA1 may pose a particular example. This receptor can be activated by a multitude of different endogenous and exogenous ligands, including several plant products. Yet, no toxin of a venomous animal has been described to our knowledge that is either activating or inhibiting this promiscuous receptor of the pain pathway. Different to TRPV1, TRPA1 is activated by a number of reactive substances that covalently modify the channel protein, thereby resulting in channel opening (Hinman et al. 2006; Macpherson et al. 2007). Reactive molecules, that only have a short half-life, may not be compatible with venoms that have to be produced and stored for longer periods of time before use and thus may lose activity fairly easily, particularly in complex venom mixtures. This is reflected in ICK peptide structures that are optimized for stability even under harsh conditions, while at the same time binding with high specificity to a range of different (membrane) receptors and ion channels.

Although ICK toxins contain a number of paired cysteines that potentially could lead to covalent modification of target receptors such as TRPA1, their oxidative strength is likely very limited, reflecting the overall ICK stability. More importantly, peptide toxins have no access to intracellular redox-sensing cysteines on TRPA1.

Whether other more reactive toxin species exist, future work will show. In this regard it is worth noting that crude venom of the platypus (*Ornithorhynchus anatinus*) harbors ingredients that can activate TRPA1 in cellular assays (David Julius and Jan Siemens unpublished observation). The venom, which is delivered by spurs located at the platypus hind limbs, is known to cause excruciating pain (Mebs 2002). In the same assay, the venom did not activate TRPV1 or TRPM8. Due to paucity of material and problematic behavior in chromatographic purification approaches—potentially reflecting reactivity of the putative compound—the venom ingredient causing TRPA1 activation was never isolated and further characterized. Whether this venom entity belongs to the peptidic toxin fraction or—more likely—to the fraction of small toxin molecules, it is thus not known to date and will require further exploration.

While chemical reactivity as a mechanism for toxin function is speculative, it is known that peptide toxins carry a vast array of modifications and their contribution to receptor specificity, avidity, as well as other toxin properties are largely unexplored.

Toxins, such as HaTx, have been instrumental in understanding biophysical parameters and helped paving the way to obtain high-resolution structural information of Kv ion channels.

For the first TRP ion channel, the capsaicin receptor TRPV1, detailed structural information is now available in the closed and the open state (Liao et al. 2013; Cao et al. 2013). For stabilizing the open conformation and deriving structural details of the gating mechanisms via cryo electron microscopy, DkTx has played a noteworthy role (see also a previous chapter in this book highlighting the TRPV1 structures).

Clearly, based on the success in the biophysical characterization of the capsaicin receptor and other ion channels, it would be highly beneficial for the TRP channel field to identify toxins with specificity for other TRP members.

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