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Receptor-targeting mechanisms of pain-causing toxins: How ow?

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Abstract

Venoms often target vital processes to cause paralysis or death, but many types of venom also elicit notoriously intense pain. While these pain-producing effects can result as a byproduct of generalized tissue trauma, there are now multiple examples of venom-derived toxins that target somatosensory nerve terminals in order to activate nociceptive (pain-sensing) neural pathways. Intriguingly, investigation of the venom components that are responsible for evoking pain has revealed novel roles and/or configurations of well-studied toxin motifs. This review serves to highlight pain-producing toxins that target the capsaicin receptor, TRPV1, or members of the acid-sensing ion channel family, and to discuss the utility of venom-derived multivalent and multimeric complexes.

1. Introduction

Venoms represent pharmacopeias of evolutionarily-honed and sophisticated toxin proteins. Through duplication and rapid divergence of toxin-encoding genes, venomous organisms have come to produce toxins that adroitly manipulate the physiology of predator and prey organisms. By virtue of the fact that they target receptors central to critical physiological processes, toxins have been useful for identifying and manipulating important signaling molecules in synaptic transmission, action potential propagation, and haemostasis (Caleo and Schiavo, 2009; Chang, 1999; Dutton and Craik, 2001; French et al., 2010; Sajevic et al., 2011; Schmidtko et al., 2010). In addition, toxins highlight portions of these receptors that define their unique properties, including ion conduction pathways of ion channels, ligand binding sites for ligand-gated receptors, and voltage-sensing domains of voltage-gated channels (Alabi et al., 2007; MacKinnon et al., 1990; Swartz and MacKinnon, 1997; Tsetlin et al., 2009). Toxins may also display secondary characteristics that enhance their potency and efficacy in unexpected ways, such as an affinity for lipids (localizing the toxin in close proximity to transmembrane receptors), a state-dependence of binding (favoring a particular conformation), or the ability to interact synergistically with other toxins (Cestele et al., 1998; Doley and Kini, 2009; Lee and MacKinnon, 2004; Milescu et al., 2007). Thus, toxins continue to reveal novel pharmacological strategies and biochemical mechanisms for manipulating specific receptors and controlling cellular function.

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Somatosensory nerve endings express a battery of receptors and ion channels that serve to transduce physical and chemical stimuli from the environment into an electrical signal of the nervous system. Individual receptors are activated by changes in temperature, pressure, oxidation state, pH, or concentrations of inflammatory signaling molecules, thereby alerting the nervous system to environmental challenges by triggering a pain response (Basbaum et al., 2009). It is not surprising, then, that these specialized receptors can be activated in the context of envenomation. Ubiquitous venom components such as phospholipases, proteases and porins (Fry et al., 2009) can damage sensory nerve endings directly, and they can also trigger the release of intracellular pro-algesic agents (e.g. ATP) from nearby cells undergoing lysis. Similarly, paralytic toxins and anticoagulant toxins can produce pain as a sequela of muscle rigidification or hemorrhagic shock, respectively. Kallikreins from cobra venom disrupt blood pressure regulation by proteolytically cleaving plasma kininogen to release bradykinin, a key mediator of inflammatory pain. In fact, cobra venom played a central role in the discovery of this pro-algesic signaling pathway (Hawgood, 1997).

Pain serves as a primary warning system for physiological distress, allowing an organism to respond to and escape from potentially dangerous stimuli. Some venoms can produce a robust perception of pain without eliciting significant tissue damage by hijacking the ion channels and receptors that directly activate somatosensory neurons (Mebs, 2002; Schmidt, 1990). Presumably, these pain-producing toxins serve to discourage threatening predators by triggering a disorienting and memorable sensory experience. Aside from the adaptive advantage they provide in nature, such toxins represent invaluable tools for understanding the molecular underpinnings of pain sensation. This utility is well-documented with regard to plant-derived small molecule irritants, such as capsaicin and menthol, which have been used to identify ion channels that normally detect changes in temperature and/or inflammatory cues (Bautista et al., 2005; Caterina et al., 1997; McKemy et al., 2002). This review will focus, instead, on venom-derived proteinaceous toxins that produce pain by activating nociceptive pathways, specifically through activation of the capsaicin receptor, TRPV1, or acid-sensing ion channels (ASICs). These toxins activate TRPV1 or ASIC receptors on sensory nerve endings at the site of envenomation, generating action potentials that propagate the toxin-initiated signals to pain processing areas in the spinal cord and brain. How these pain-producing toxins are able to selectively and potently activate their target receptors cannot be illustrated without considering the molecular compositions of the toxins, which exemplify biochemical strategies that venom proteins employ to produce their profound effects.

2. TRPV1 toxins: feel the burn

TRPV1, a member of the transient receptor potential (TRP) superfamily of excitatory ion channels, was initially identified as the receptor for capsaicin, the pungent ingredient in chili peppers (Caterina et al., 1997). TRPV1 is expressed predominantly by nociceptors (peripheral sensory neurons that respond to painful stimuli), where it is activated by a variety of noxious signals, including high temperature, acidic pH, and inflammatory second-messenger cascades (Tominaga et al., 1998). TRPV1 is chiefly found in nerve fibers enervating the skin and visceral organs, although it is also expressed highly within vasodilatory smooth muscle cells in thermoregulatory tissues, and extremely low levels of expression can be detected in limited brain regions (Cavanaugh et al., 2011). Knockout mice lacking TRPV1 show dramatically reduced hypersensitivity to heat during inflammation (Caterina et al., 2000; Davis et al., 2000), and TRPV1 has become a major focus in pharmaceutical efforts to attenuate inflammatory, neuropathic, and cancer-related pain(Culshaw et al., 2006; Gavva et al., 2005; Ghilardi et al., 2005; Pomonis et al., 2003).

In addition to members of the *capsicum* genus (i.e. chili peppers), a variety of species have evolved toxins that directly activate TRPV1 (Fig. 1) (Cromer and McIntyre, 2008). The

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succulent *Euphorbia resinifera* and certain other species of spurge produce the small molecule resiniferatoxin, which is a much more potent and longer-lasting TRPV1 agonist than capsaicin itself (Appendino and Szallasi, 1997; Caterina et al., 1997). As many venomous bites and stings also produce a painful burning sensation, it stands to reason that some venoms may achieve these agonizing effects by activating TRPV1. Indeed, three homologous toxins have been identified from the venom of *Psalmopoeus cambridgei*, a tarantula from the West Indies, that produce robust pain and inflammation through activation of TRPV1 (Siemens et al., 2006). The presence and spacing of six cysteine residues in each toxin sequence characterizes them as inhibitor cysteine knot (ICK) toxins. These toxins were named 'vanillotoxins' (VaTx1-3) to reflect their ability to activate TRPV1 with similar efficacy as the vanilloid compounds capsaicin and resiniferatoxin. A distantly related Chinese tarantula, *Ornithoctonus huwena*, also produces a potent TRPV1-activating toxin that bears little sequence similarity to VaTx1-3, indicating that multiple tarantula species have developed toxins that target this important receptor through convergent evolution (Bohlen et al., 2010). Interestingly, the *O. huwena* toxin consists of two tandemly-repeated ICK motifs that produce long-lasting TRPV1 activation through a bivalent mechanism, earning it the name 'double-knot toxin' (DkTx). VaTx1-3 and DkTx provide unique insights into venom evolution and diversification, which will be described in the following sections.

TRPV1 can be activated by toxins directly, as described above, but it can also be sensitized indirectly through the recruitment of modulatory second-messenger cascades (Fig. 1). Pro-inflammatory mediators such as bradykinin, ATP, or prostaglandins activate G-protein coupled receptors (GPCRs) that enhance TRPV1 activity by altering its phosphorylation state or lipid surroundings (Nieto-Posadas et al., 2012; Tominaga and Tominaga, 2005). As such, TRPV1 can be sensitized by venom components such as kallikreins, phospholipases, and proteases that do not interact directly with the receptor. The multitudinous effects of these molecules need not be considered from a TRPV1-centric viewpoint, but in some cases TRPV1 is necessary for a toxin's pro-nociceptive effects despite being an indirect target. For example, a protein toxin from frog (*Bombina variegata*) skin, Bv8, produces hyperalgesia by activating PKCε-coupled prokineticin receptors, which leads to phosphorylation and sensitization of TRPV1 (Vellani et al., 2006); mice lacking TRPV1 require 100-fold higher doses of toxin to achieve the hyperalgesia observed in wild-type animals (Negri et al., 2006). Homologues of Bv8 have been found in other frog, fish, monitor lizard, and spider species, suggesting that many venoms sensitize nociceptors through this mechanism (Fry et al., 2006; Negri et al., 2007; Schweitz et al., 1990; Szeto et al., 2000; Wen et al., 2005). The tentacle extracts of several cnidarians species (the lion's mane jellyfish, a species of box jellyfish, an anemone, and the Portuguese Man o' War) as well as the polyether toxins gambierol and brevetoxin (the central toxins of ciguatera poisoning) also potentiate activation of TRPV1 by capsaicin, but likely do so through direct interactions with the channel or the cell membrane (Cuypers et al., 2006; Cuypers et al., 2007).

TRPV1-inhibiting toxins have been identified in two venoms, although the physiological significance of these compounds in the context of envenomation remains enigmatic. APHC1, a peptide isolated from nematocyst extract of the sea anemone *Heteractis crispa*, partially inhibits capsaicin-evoked TRPV1 responses and reduces pain-related behaviors in mice challenged with heat or capsaicin (Andreev et al., 2008). Two small molecule compounds (AG489 and AG505) from the venom of the funnel web spider *Agelenopsis aperta* were also found to inhibit TRPV1, but they had been previously identified as non-specific ion channel blockers, and their interaction with TRPV1 may represent an off-target effect (Kitaguchi and Swartz, 2005). On the other hand, several venom toxins are known to produce analgesic effects, and these may play adaptive roles that we still do not fully understand (Beeton et al., 2006).

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3. The two-faced vanillotoxins: acquiring a new identity

The TRPV1-activating vanillotoxins present a rare case-study of how one of the most commonly found scaffolds in venom toxins, the ICK motif, can be adapted to novel functions. The disulfide-rich ICK motif allows relatively short toxin sequences (typically 25-45 amino acids) to adopt conformationally-constrained and stable tertiary structures (Daly and Craik, 2011). ICK toxins fulfill a broad range of different functions that have been acquired through a process of gene duplication and rapid diversification (Conticello et al., 2001; Kordis and Gubensek, 2000; Sollod et al., 2005). For example, VaTx1-3 are 53-82% identical to one another, but they embody distinct stages of functional specificity: VaTx1 both activates TRPV1 and inhibits the voltage-gated potassium channel $K_v2.1$ within the same concentration range, whereas VaTx2 and VaTx3 exhibit progressively lower potency against $K_v2.1$ as well as higher potency for TRPV1 (Siemens et al., 2006). This apparent progression of functionality is also reflected in toxin sequence alignments, which show that VaTx1 is most similar to K_v inhibitor toxins from related tarantulas (such as the *Heteroscodra maculata* toxin HmTx1), and that VaTx1 and VaTx3 share less similarity with one another than either shares with VaTx2 (Escoubas et al., 2002; Siemens et al., 2006).

Interestingly, VaTx1 recognizes distinct regions of K_v and TRP channels, despite the fact that these two channel families share a similar overall architecture. Both K_v and TRP channels are likely tetrameric and contain six transmembrane helices per monomer, with the fifth and sixth transmembrane helices comprising the core pore-forming domain of the tetrameric complex (Hoenderop et al., 2003; Kedei et al., 2001; Long et al., 2005; Voets et al., 2002). This pore-forming domain is the region that specifies TRPV1 sensitivity to vanillotoxins (Bohlen et al., 2010). In contrast, VaTx1 inhibits $K_v2.1$ through interactions with the voltage-sensing domain (the third and fourth transmembrane helices), much like the well-studied gating-modulator toxins of the voltage-gated channel family (Bohlen et al., 2010; Catterall et al., 2007; Swartz, 2007). Remarkably, then, VaTx1 has evolved to interact with two distinct regions of K_v and TRP channels, presumably by presenting two distinct (although perhaps overlapping) pharmacophore ‘faces’ to manipulate its two channel targets. In comparison, VaTx2 and VaTx3 demonstrate a diminished functionality of the $K_v2.1$ pharmacophore in favor of refining the TRPV1-binding surface. Indeed, many toxins have acquired fundamentally different roles over evolutionary time, such as the Kunitz family of toxins, which were originally recruited to venoms as protease inhibitors, but have since acquired the capacity to block voltage-gated potassium channels (Fry et al., 2009). The transition between these functionalities can be appreciated in the bifunctional huwentoxin-XI, in which both a trypsin-inhibiting surface and a K_v channel-inhibiting surface are displayed on opposite faces of the toxin (Yuan et al., 2008). In line with this theme, the vanillotoxins could be considered as preserved intermediates in a progression between paralyzing and pain-inducing toxin functionalities, although the binding surfaces that dictate their graded specificity remain to be identified.

4. Multivalent toxins: two heads are better than one

While the vanillotoxins present an interesting example of a well-known phenomenon (the conversion of a toxin's activity profile from one function to another), the TRPV1-activating toxin from *Ornithoctonus huwena* venom (DkTx) illustrates a less-appreciated venom strategy. The full-length DkTx sequence consists of two highly homologous ICK motifs in tandem (see Fig. 2), apparently the result of a gene duplication event that placed both the original and duplicated motifs in the same open reading frame. The resulting toxin can be proteolytically cleaved into two separately-folded ICK toxins, each of which activates TRPV1 when applied on its own. However, the tandemly-conjoined full-length toxin has significantly higher potency and a dramatically longer-lasting association with TRPV1 than either of the separated ICK domains has on its own, far beyond that which would result from

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their additive contributions (Bohlen et al., 2010). Thus, this aggressive tarantula has developed an enhanced and near-irreversible pain-producing ligand through duplication of a toxin-encoding gene.

Combining multiple copies of a molecule into a single compound to heighten its effectiveness is a well-known strategy in pharmaceutical chemistry (Choi, 2004). Whether targeting many receptors across a viral or cellular surface (polyvalency), targeting multiple distinct binding sites on a receptor (hetero-multivalency), or targeting identical binding sites on multiple subunits of a multimeric complex (homo-multivalency), this approach relies on tethering multiple ligands together such that they bind to multiple receptor sites simultaneously. In theory, physically connecting monovalent ligands can strengthen binding affinity enormously. A trivalent derivative of the antibiotic vancomycin approaches ideality in this regard; it binds to a trivalent ligand (a molecule displaying three D-Ala-D-Ala dipeptides) with 10^{10} higher affinity than monovalent vancomycin (Rao et al., 1998). The enthalpic contribution to monomer binding is nearly tripled in the trivalent vancomycin, and this additive contribution to binding energy (ΔH^{mono} = -50.2 kJ mol^{-1}, ΔH^{tri} = -167 kJ mol^{-1}) results in a multiplicative improvement in binding affinity ($K_d^{mono} = 1.6 \times 10^{-6}$ M, $K_d^{tri} = 4 \times 10^{-17}$ M) (Rao et al., 1998). The measured dissociation constant for the trivalent vancomycin falls short of the theoretical prediction (the K_d of the monomer taken to the third power) due to an entropic penalty associated with conformational constraint. More generally, the energetic effects of multivalent molecule binding events are difficult to predict due to restricted access to binding sites and steric effects, but multivalent ligands commonly exhibit 10- to 1000-fold increases in binding affinity compared to their monovalent counterparts (Choi, 2004).

Multivalent ligands also typically out-perform their monovalent counterparts in persistence of action. The long-lasting nature of multivalent ligands is well-illustrated by a synthetic cGMP dimer (two cGMP molecules tethered by a PEG-spacer) that was designed as a bivalent agonist for the homotetrameric CNG channel (Kramer and Karpen, 1998). When one cGMP moiety (one half of the bivalent ligand) has bound, the second is restricted to a very small volume prescribed by the linker length. Because the second cGMP moiety is restricted to so small of a volume (on the order of femtoliters), even a single molecule represents an extremely high effective concentration, driving its very rapid association with a second nearby subunit of the tetrameric channel. A quantitative model predicts that, since both cGMP moieties must dissociate for the bivalent molecule to be fully liberated from its channel target, the dissociation rate of the bivalent ligand (k_{off}^{bi}) is proportional to the dissociation rate of the monomer (k_{off}^{mono}) multiplied by the probability that the second monomer is unbound, and a statistical factor of two: $k_{off}^{bi} = 2k_{off}^{mono} K_d^{mono}/(K_d^{mono} + C_{eff})$. The effective concentration of the tethered second cGMP molecule, C_{eff}, can be estimated by assuming it is restricted to an evenly-populated hemisphere with radius equal to the bivalent ligand's linker length: $C_{eff} = 1/(N_A) \times 1/(2/3\pi r^3)$ (see Fig. 2) where r is the linker length in decimeters and N_A is Avogadro's number (Kramer and Karpen, 1998). More refined models have been developed to incorporate random walk statistics for specific polymer chains and non-hemispheric excluded volume effects (Gargano et al., 2001; Krishnamurthy et al., 2007). Although the extremely slow dissociation rate observed with multivalent ligands typically excludes meaningful measures of k_{off}^{bi}, the theory illustrates the extreme kinetic enhancements of multivalent ligands over their monovalent counterparts. Notably, multivalent interactions undergo accelerated dissociation in the presence of very high concentrations (approaching C_{eff}) of monovalent ligand, which competes for occupancy of the second (or third, or fourth, etc.) binding site (Kramer and Karpen, 1998; Smith et al., 2006).

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Multivalent (non-toxin) ligand design has been explored for numerous receptors of pharmaceutical interest, including cholinergic receptors (Christopoulos et al., 2001; Rosini et al., 1999), adrenergic receptors (Kizuka and Hanson, 1987), opioid receptors (Portoghese et al., 1988), serotonin receptors (Leboulluec et al., 1995), voltage-gated calcium channels, voltage-gated sodium channels (Joslyn et al., 1988; Smith et al., 2006), and a diversity of surface receptors from infectious viruses and bacteria (Choi, 2004). Natural toxins target a broad spectrum of multimeric receptors and have been of great use in basic research as well as therapeutic endeavors, where their pharmacological profiles have occasionally made them useful pharmaceuticals (Lewis and Garcia, 2003; Terlau and Olivera, 2004). Although some toxins have been extensively modified to improve specificity, potency, and/or bioavailability (Craik and Adams, 2007), multivalent strategies for improving toxin effectiveness remain almost entirely untapped. One exception is the utilization of a thrombopoieten receptor-binding peptide that has been incorporated into an ICK-toxin-like scaffold. When two of the 'miniproteins' are covalently crosslinked together, they produce a bivalent ligand that promotes dimerization and consequent activation of the thrombopoieten receptor (Krause et al., 2007). Considering the abundance of multimeric receptors that are targeted by natural toxins, there remains great potential for designing more potent and specific multivalent ligands for these receptors. Some targets (for example the voltage gated sodium channel family) contain several distinct toxin binding sites that alter channel gating, which could potentially be engaged simultaneously with hetero-multivalent ligands (Catterall et al., 2007; Smith et al., 2006). The advantage of subtype specificity found in other toxins, for example K_v channel inhibitor toxins (Mouhat et al., 2008), could in principle be exploited to generate novel hetero-multivalent ligands that are specific for hetero-multimeric receptor complexes.

Outside of pharmaceutical science, multivalency can be readily observed throughout biology, most familiarly in the form of immunoglobulins, surface-exposed lectins, and DNA-binding proteins (Choi, 2004). It is likely that venomous creatures also employ multivalency in the toxins they produce, as they stand to benefit from the high affinity and slow dissociation rates of multivalent toxins and express gene-duplication machinery to foster evolution of these molecules (Kordis and Gubensek, 2000). The extreme case of sarafotoxin highlights this potential for multivalent toxin production in venom glands: the burrowing asp *Atractaspis engaddensis* produces a sarafotoxin precursor peptide that contains twelve repeats of the toxin sequence in tandem (Ducancel et al., 1993).

While in the case of sarafotoxin (and several other tandemly-repeated toxin precursors) active toxin monomers are proteolytically liberated from their precursor post-translationally, other venoms contain intact, functional multi-domain toxins that may engage in multivalent interactions. DkTx presents a clear case where multiple TRPV1-binding toxins are tethered to produce a ligand that is more potent and longer-lasting than its monovalent peers (VaTx1-3 and the separated ICK domains of DkTx), presumably resulting in a heightened pain response in envenomated victims (Bohlen et al., 2010). Additionally, a toxin containing two tandem ICK motifs was recently discovered in venom from the yellow sac spider (*Cheiracanthium punctorium*) (Vassilevski et al., 2010). The cytolytic function of this *C. punctorium* toxin likely contributes to the local pain and erythema that results from envenomation, but whether this occurs by a bivalent mechanism is, as yet, unknown. Transcriptomic analysis of the banded Gila monster (*Heloderma suspectum cinctum*) venom gland identified a toxin, helofensin, which contains four tandem β-defensin repeats with no apparent protease cleavage site in the short linkers connecting them, and may therefore function as a multivalent toxin (Fry et al., 2010). Finally, venomous secretions from ticks contain toxins that are comprised of multiple Kunitz-type domains (Mans et al., 2008; van de Locht et al., 1996), although these tandem domains likely serve to simultaneously inhibit multiple coagulation proteases locally, as in the case of the mammalian multi-Kunitz domain tissue factor pathway inhibitor, TFPI (Lwaleed and Bass, 2006). Interestingly, the

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anticoagulant rhodnin from the kissing bug, *Rhodnius prolixus*, is also formed by two tandemly-repeated Kazal-type domains (Friedrich et al., 1993). While the toxins described above are examples of tandemly-repeated motifs within a single peptide chain, other noncovalently- or disulfide-linked toxin multimers have been identified with properties that point to a multivalent interaction with their receptors. Such multi-protein toxin complexes will be discussed below.

5. ASIC toxins: feeling sour

Members of the ASIC family of ion channels contribute to the responsiveness of nociceptive nerve fibers to acidosis. Acidic pH activates subsets of neurons found in somatosensory ganglia (Krishtal and Pidoplichko, 1980), and the response profile depends on the severity of acidification. While TRPV1 is chiefly responsible for slowly-desensitizing currents elicited by extreme acidification (pH < 6) (Caterina et al., 2000; Davis et al., 2000; Leffler et al., 2006), ASIC channels produce relatively rapidly-desensitizing excitatory currents that result from milder acidic insults (Drew et al., 2004; Poirot et al., 2006), within a pH range that is typical of tissue acidosis during ischemia or some types of inflammation. The large extracellular domain of ASICs allow them to respond to extracellular protons with high sensitivity and cooperativity, presumably via interaction with multiple acidic amino acids whose pKa has been tuned by neighboring residues. Functional channels are made up of homotrimeric or heterotrimeric assemblies of distinct ASIC subtypes (ASIC1-3) or their splice variants (ASIC1a/1b and ASIC2a/2b), with various subunit compositions forming channels with different pH-sensitivities and desensitization rates (Hesselager et al., 2004; Jasti et al., 2007).

While ASICs may be activated briefly during envenomation (due to the venom's acidic pH or as a consequence of tissue inflammation), three toxins have been identified that modulate ASICs directly (Fig. 1). Two of these act as potent and effective ASIC inhibitors. APETx2 from the sea anemone *Anthopleura elegantissim* inhibits ASIC3 homomeric channels as well as ASIC3-containing heteromers (Diochot et al., 2004). The same tarantula (*P. cambridgei*) that produces the TRPV1-activating toxins VaTx1-3, also expresses an ASIC inhibitor, PcTx1, which exhibits high specificity for homomeric channels formed by the ASIC1a splice variant (Escoubas et al., 2000). While ASIC transcripts have been detected in somatosensory ganglia and a range of other tissues, including brain, bone, bladder, retina, inner-ear, tongue, testis, and lung (Lingueglia, 2007), it is not clear what selective advantage these inhibitory peptide toxins confer, perhaps reflecting our limited understanding of the physiological roles played by ASICs in neural and non-neural systems. Nevertheless, APETx2 and PcTx1 toxins have been useful tools for pharmacologically isolating ASIC3-containing channels at sensory nerve-endings, or ASIC1a channels in the spinal cord and brain, supporting a role for these channel subtypes in pain or fear and ischemia, respectively (Deval et al., 2010; Deval et al., 2011; Mazzuca et al., 2007; Pignataro et al., 2007; Ziemann et al., 2009).

In contrast, the venom of the Texas coral snake (*Micrurus tener tener*) produces pain through *activation* of ASIC channels. The purified ASIC-activating component of the venom, MitTx, is sufficient to elicit robust nocifensive behaviors (Bohlen et al., 2011), and while it may also have effects in other tissues, MitTx is likely responsible for the lasting pain experienced by victims of coral snake envenomation (Morgan et al., 2007; Nishioka et al., 1993). Indeed, while ASICs activated by acidic pH typically desensitize completely over the course of a few seconds, MitTx-evoked responses are largely nondesensitizing and therefore carry far more current than would be produced even by severe acidification. MitTx preferentially activates ASIC1-containing channels, although it has profound sensitizing effects on ASIC2a homomers and can activate ASIC3 at high concentrations. Interestingly, the behavioral response to MitTx injection is completely absent (at least at the doses tested) in

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ASIC1 knockout mice, highlighting that activation of the ASIC1 subtype is capable of driving pain-related behaviors (Bohlen et al., 2011). In this case, the pain-producing toxin illustrates a receptor-targeting mechanism by which snakes produce pain, while highlighting a role for ASIC1 in pain sensation.

Unlike the other toxins thus far described, MitTx is formed by two protein subunits, MitTx-α and MitTx-β. Each subunit contains a characteristic pattern of cysteines that places it into a well-characterized toxin family; MitTx-α is a Kunitz-type toxin and MitTx-β is a phospholipase-A2 (PLA2)-like toxin. However, the MitTx subunits clearly deviate from the canonical protease-inhibiting and phospholipase functionalities of these toxin families, again illustrating how venoms duplicate and dramatically alter a core set of structural motifs to take on novel functions. MitTx-α and MitTx-β form a high-affinity 1:1 complex, and neither subunit demonstrates functional effects on its own (Bohlen et al., 2011). Why both toxin subunits are required for activation of ASICs remains unknown, but several hypotheses can be generated by considering other known toxin complexes.

6. Multi-component toxins: strength in numbers

Venoms typically contain dozens to thousands of molecules with varying structures and diverse functional effects. The resident toxins do not all operate independently however, as multiple toxins within a particular venom often conspire by targeting different steps of the same physiological signaling pathway. For example, fish-hunting cone snails produce a so-called “motor cabal” of toxins, which includes voltage-gated sodium channel inhibitors that disrupt action potential propagation on the motor neuron, voltage-gated calcium channel inhibitors that disrupt vesicular release from the motor neuron, and acetylcholine receptor inhibitors that disrupt synaptic transmission (Terlau and Olivera, 2004). In addition to complementing the functional effects of other venom components, toxins can form direct multimeric complexes that demonstrate emergent properties. While multimeric toxins have been studied chiefly in snake venom, they have also been identified in a range of venomous species, including cone snails, scorpions, and ants (Doley and Kini, 2009; Loughnan et al., 2006; Pluzhinikov et al., 1994; Zamudio et al., 1997).

What advantages do multimeric toxins provide over their monomeric counterparts? While venom proteins typically conform to a relatively small number of stable structural motifs, multimerization combinatorially expands the number of scaffolds that can be employed for target recognition, allowing for improved tissue localization and/or receptor recognition (see Fig. 3). Indeed, toxin complexes often demonstrate novel activities that are completely absent from the singular subunits. An acetylcholine receptor inhibitor from cobra venom, α-cobratoxin, utilizes cysteine residues that are dispensable for folding to form various disulfide-linked dimers. The α-cobratoxin homodimer demonstrates new functionality, potently inhibiting α3β2 acetylcholine receptors, a receptor subtype that is insensitive to monomeric α-cobratoxin in the same concentration range (Osipov et al., 2008). Although the dimeric species of α-cobratoxin are low in abundance and the mechanism by which dimerization changes subtype specificity remains undefined, α-cobratoxin dimers illustrate how functional toxins can become incorporated into multimeric complexes so as to expand the repertoire of a given venom's effects.

Similarly, the heterodimeric MitTx complex is able to activate ASICs, but the individual subunits have no apparent functional effects on their own. MitTx has an unusual molecular makeup (one PLA2-like subunit non-covalently associated with one Kunitz-type subunit), but the well-studied heterodimeric toxin β-bungarotoxin and its isoforms have a very similar composition and may be informative in considering the contribution of MitTx subunits. Mature β-bungarotoxin is formed by two disulfide-linked protein chains, one active PLA2 (chain-A) and one Kunitz-type toxin (chain-B) (Kondo et al., 1978). Chain-B binds and

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inhibits voltage-gated potassium channels (Dreyer and Penner, 1987), and has been proposed to act as a chaperone to localize the phospholipase activity of chain-A to presynaptic terminals, focally compromising membrane integrity and facilitating synaptic release. In the case of β-bungarotoxin, the contribution of each chain has not been unambiguously defined, and its mechanism of action might be more complicated (see (Rowan, 2001)). Such a chaperoning mechanism has been demonstrated for crotoxin (the first multimeric toxin to be identified in venoms), whose two subunits are approximately ten-times more lethal when applied together than alone, despite the fact that they undergo a necessary dissociation step upon binding to synaptic membranes (Bon et al., 1979; Hendon and Tu, 1979). These toxins illustrate how a chaperone-like subunit could enhance the activity of a multimeric toxin.

Taicatoxin, an inhibitor of voltage-gated calcium and potassium channels from venom of the Australian taipan (*Oxyuranus scutellatus scutellatus*), is also a non-covalent complex of PLA2 and Kunitz type subunits (Brown et al., 1987; Possani et al., 1992). However, taicatoxin is a hexameric complex with one PLA2 subunit, four Kunitz protein subunits, and one subunit belonging to the three-finger toxin family (Doorty et al., 1997; Possani et al., 1992). A high-resolution structure of this large complex and the contributions of the individual subunits have yet to be fully determined, but this elaborate toxin highlights the diversity of structures and functions that can arise from multimerization using common toxin structural motifs.

Another functional enhancement that may drive formation of higher-order toxin complexes is multivalency, similar to what was described for DkTx above. This scenario can be appreciated in C-type lectin-like proteins (CLPs) from snake venom, which form dimeric subunits that further aggregate into higher-order assemblies (Doley and Kini, 2009). In the case of the particularly large and well-characterized CLP, convulxin, two disulfide-linked octamers assemble back-to-back, allowing the toxin to bind to multiple receptor molecules on each of two adjacent platelets, thereby forming a high-avidity multivalent complex that promotes platelet agglutination (Horii et al., 2009). Additionally, several other multimeric toxins are likely to take advantage of a multivalent strategy, although a multivalent mode of action has not been demonstrated for them directly. Con-ikot-ikot is a toxin from *Conus striatus* venom that prevents desensitization of AMPA receptors. The toxin was found to have a unique ‘dimer of dimers’ composition, where two disulfide-linked homodimers interact with each other non-covalently to form a tetrameric complex (Walker et al., 2009). Considering the tetrameric nature of the glutamate receptor target, the close apposition of the gating modules found on each of the channel's four subunits (Sobolevsky et al., 2009), and the slow washout of toxin-evoked effects (Walker et al., 2009), con-ikotikot may act as a bivalent or tetravalent glutamate receptor ligand. Along a similar line, two acetylcholine receptor inhibitors from snake venoms, haditoxin and irditoxin, exist as disulfide-linked dimers and exhibit near-irreversible inhibition of their pentameric target receptors (Pawlak et al., 2009; Roy et al., 2010).

Multimeric toxins may also demonstrate improved pharmacological specificity simply because multiple subunits present more potential contact sites to strengthen interactions with their target. Along a similar vein, binding of two toxin subunits may result in conformational changes of each subunit's target-recognition surface. The second scenario seems to be the case for the conantokin family of NMDA receptor antagonists from *Conus* venoms, which can form dimers in the presence of divalent cations (Dai et al., 2004). Structures have been solved for monomeric and dimeric forms, revealing side-chain rotations opposite to the dimerization surface (Cnudde et al., 2010; Cnudde et al., 2007); additionally, synthetic stabilized dimers demonstrate different NMDA receptor subtype specificity compared to the

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monomeric form, suggesting that the conformational changes induced by dimerization are functionally significant (Dai et al., 2007).

In summary, multimeric toxin subunits can act as chaperones, contribute to multivalent binding events, and adjust or expand the target-recognition/active site of partner subunits. A wide variety of multimeric forms have been identified to date, and unique complexes with unprecedented functional roles likely remain to be discovered. Still, the properties of well-studied complexes can be used to generate testable hypotheses about the functional role of individual subunits in other multimeric toxins, or to guide *in vitro* synthesis of new, highly potent and selective pharmacophores.

7. Conclusion

Envenomation can sometimes induce euphoric catalysis or a relatively aloof hemorrhagic state, but more typically, venoms produce pronounced pain and inflammation (Chahl and Kirk, 1975). While in some cases, pain certainly occurs as a side-effect of tissue damage, the existence of toxins that target TRPV1, a receptor found predominantly in nociceptors, suggests that noxiousness is an end in itself for a subset of venom proteins, representing a likely mechanism to avert predation (Siemens et al., 2006). In addition to the cases described above, there are many venoms that cause intense or characteristic pain for which the responsible toxins have not been identified. The Schmidt pain index, a metric describing the intensity of suffering that results from bites and stings from various organisms, highlights several species (e.g. the tarantula hawk and the bullet ant) that can evoke intense pain, but which lack a molecular description of how these severe effects are produced (Schmidt, 1990). Pain is also the principle symptom of envenomation from a number of fishes (weeverfish, lionfish, scorpionfish, and stingray) and a significant effect of centipede and Gila monster venoms (Mebs, 2002; Strimple et al., 1997; Undheim and King, 2011). However, the toxins and targets responsible for the sensations caused by these understudied venoms are still unknown. The diversity of painful sensations that accompany different types of envenomation (e.g. burning pain versus electrical pain) suggests that distinctive strategies have evolved for manipulating the somatosensory system.

Venoms can be exploited to enhance our understanding of normal and pathological pain sensation, and reciprocally, function-based screening serves as a complementary approach to proteomic and transcriptomic efforts for understanding toxin diversity. The unusual compositions of the multivalent DkTx and the multimeric MitTx stand as testament to this claim, representing novel toxins that would likely have been overlooked by conventional transcriptomic and proteomic efforts. Understanding the function and targets of these (and other) toxins provides a light by which their molecular intricacy can be appreciated.

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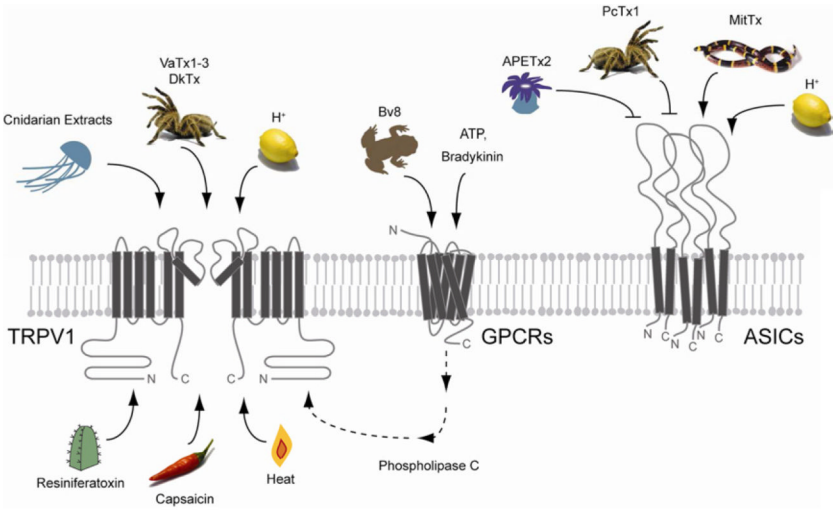

Figure 1. Toxins targeting somatosensory receptors

(Left) Two of the four TRPV1 subunits are shown schematically with six gray bars representing transmembrane helices. 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. (Right) ASICs are trimeric channels with each subunit containing two transmembrane domains. ASICs are activated by pain-causing toxins from some coral snake venoms as well as acidic pH. Two ASIC-inhibiting toxins have been identified from tarantula and sea anemone, respectively.

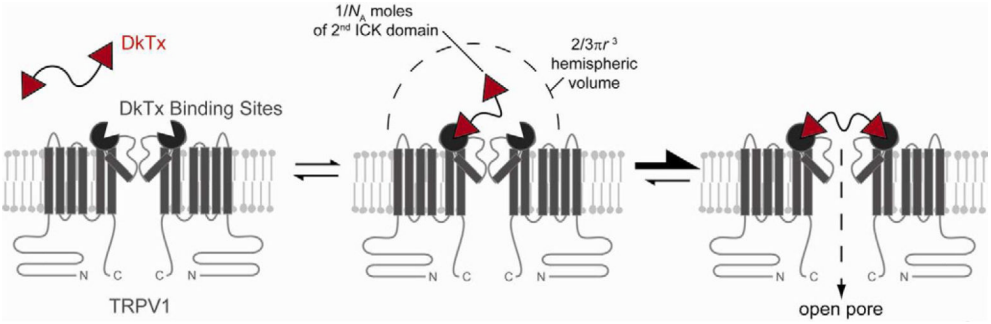

Figure 2. Strengthened binding of a bivalent ligand, DkTx, to its receptor, TRPV1

(Left) DkTx activates TRPV1 by binding to the extracellular region near the fifth and sixth transmembrane domains (illustrated as in Figure 1). (Middle) Once one ICK domain (red triangle) has bound to the receptor, the second domain is restricted to a minute volume determined by the length of the toxin's linker region. The high local concentration of the second domain near the binding site of an adjacent subunit results its rapid binding. N_A represents Avogadro's number and r represents the length of the linker between ICK domains. (Right) Both ICK domains must dissociate for the channel to remain closed, but when one domain dissociates, it is likely to rapidly bind again before the second domain escapes.

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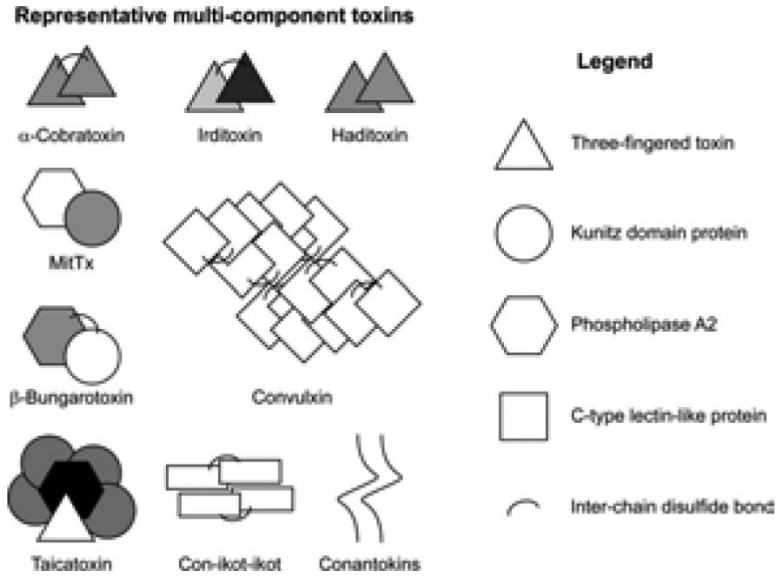

Figure 3. Multimeric venom toxins

A diversity of homo- and hetero-multimeric protein toxins have been identified from venom, either as non-covalent or disulfide-linked complexes. A number of common toxin structural scaffolds (Legend, right) can be recruited to assemble toxin complexes with novel functions.

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