Published in final edited form as:

Toxicon. 2014 October ; 0: 45–54. doi:10.1016/j.toxicon.2014.06.014.

A Family of Excitatory Peptide Toxins from Venomous Crassispine Snails: Using Constellation Pharmacology to Assess Bioactivity

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Abstract

The toxinology of the crassispine snails, a major group of venomous marine gastropods within the superfamily Conoidea, is largely unknown. Here we define the first venom peptide superfamily, the P-like crassipeptides, and show that the organization of their gene sequences is similar to conotoxin precursors. We provide evidence that one peptide family within the P-like crassipeptide superfamily includes potassium-channel (K-channel) blockers, the κ P-crassipeptides. Three of these peptides were chemically synthesized (cce9a, cce9b and iqi9a). Using conventional electrophysiology, cce9b was shown to be an antagonist of both a human $K_{V1.1}$ channel isoform (Shaker subfamily of voltage-gated K channels) and a *Drosophila* K-channel isoform. We assessed the bioactivity of these peptides in native mammalian dorsal root ganglion neurons in culture. We demonstrate that two of these crassipeptides, cce9a and cce9b, elicited an excitatory phenotype in a subset of small-diameter capsaicin-sensitive mouse DRG neurons that were also affected by κ J-conotoxin p114a, a blocker of $K_{V1.6}$ channels. Given the vast potential complexity of heteromeric K-channel isoforms, this study demonstrates that the crassispine venoms are a potentially rich source for discovering novel peptides that can help to identify and characterize the diversity of K-channel subtypes expressed in native neurons and other cell types.

INTRODUCTION

One of the most biodiverse invertebrate lineages in the marine environment encompasses the venomous marine snails in the superfamily Conoidea, a group that by current estimates

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comprises more than 10,000 species (Bouchet et al., 2011; Olivera et al., 2014). Among these are the crassispirine neogastropods, a group long regarded as a subfamily of the family Turridae s.l. (McLean, 1971; Powell, 1966; Taylor et al., 1993), but in some more recent taxonomic work, included as a major clade within the family Pseudomelatomidae (Bouchet et al., 2011), an assemblage of venomous marine gastropods that encompasses more than 50 recent genera. These conoideans (which we will refer to as crassispirines or crassispirine snails) have generally been understudied and their toxinology is largely unknown. However, some crassispirine species were shown to possess hypodermic radular teeth, notably similar to those of cone snails (Kantor et al., 1997), suggesting a similar mechanism of prey envenomation.

In the Indo-Pacific, crassispirine snails are mostly collected offshore (in contrast to the Atlantic and Eastern Pacific, where a significant number of species live in shallow water). In recent years, access to live specimens of crassispirines from the Indo-Pacific has been provided by shell-gathering fishermen from the Philippines, who invented two novel methods for collecting snails from off-shore habitats. One such method is a gill net, a long fine-mesh net that is laid out overnight in deep water, typically at depths of 30-150m, and lifted before dawn. Many snails that happen to be on the nets as they are lifted become entangled within the net (for this reason, these are also called “tangle nets”) (Ng et al., 2009). A second method is to tie together old discarded fishing nets into a tight bundle, drop the bundle at the bottom and leave it for a period of two weeks to 6 months. These bundled nets, called *lunun-lunun* (Seronay et al., 2010), then become an ecosystem settled by small marine invertebrates. The richness of the net community that develops depends on the density of planktonic larvae that settle in the net, which is largely determined by the prevailing currents. When the nets are lifted, a large assortment of small molluscs (and other marine invertebrates) can be collected. Because of these innovations, routine access has been gained to live venomous crassispirine snails, making the first direct observations of their predatory behavior possible, as demonstrated in this study. In this paper, we describe the results of our studies on two species of crassispirine snails, *Crassispira cerithina* (Anton, 1839), a species that is routinely harvested from the *lunun-lunun* nets described above, and *Inquisitor intertincta* (Smith, 1877), which is generally collected using the gill net method.

The initial results of our toxinological investigation of these two species are presented here. The first family of venom peptides from the crassispirines, the κ P-crassipeptides, has been defined by these studies. We also have utilized both conventional electrophysiology, as well as constellation pharmacology, a new approach for elucidating the biological activity of such families of venom peptides. In this study, we show that by simultaneously assaying a broad spectrum of receptors and ion channels expressed in a heterogeneous mixture of different cell types (Smith et al., 2013; Teichert et al., 2014a; Teichert et al., 2014b; Teichert et al., 2012a; Teichert et al., 2012b), we are able to assess bioactivity and provide an initial definition of the potential selectivity of newly-discovered venom peptides.

MATERIALS AND METHODS

Phylogenetic analysis of crassispine snails

Using the SeaView package (Gouy et al., 2009), we aligned individual 12SrRNA, 16SrRNA, COI, sequences with Muscle (Edgar, 2004). We compared models of sequence evolution using PhyML (Guindon et al., 2010) and inferred maximum likelihood trees using the optimal GTR+I+G model (Tavaré, 1986) for the concatenated sequences of all three genes. Approximate Likelihood Ratio test (aLRT) statistics to provide branch length support for the trees (Guindon et al., 2010).

In addition, we used MrBayes (Huelsenbeck et al., 2001; Ronquist and Huelsenbeck, 2003) to infer model parameters and the tree using optimal partitioning for the concatenated sequences of all three genes. Each analysis comprised two simultaneous runs with four chains each for enough generations to reduce the average standard deviation of the split frequencies below 0.01 (about 17 million generations). Trees and parameters from the first 25% of the generations were discarded (the burn in) after completion of the MCMCMC (Metropolis Coupled Monte Carlo Markov Chain) search. Posterior Probabilities on the consensus tree provide branch support.

Identification and sequencing of cDNA clones encoding P-like crassipeptides

cDNAs were prepared by reverse transcription of RNAs from *Crassispira cerithina* and *Inquisitor intertincta*, venom ducts as previously described (Watkins et al., 2006). The resulting cDNAs were used as templates for polymerase chain reaction (PCR) using oligonucleotides corresponding to the conserved 5' and 3' UTR sequences of P-like prepropeptides. The resulting PCR products were purified using the High Pure PCR Product Purification Kit (Roche Diagnostics, Indianapolis, IN).

The eluted DNA fragments were annealed to pNEB206A vector using the USER Friendly Cloning kit (NewEngland BioLabs, Inc., Beverly, MA) following manufacturer's suggested protocol and the resulting products transformed into DH5 α competent cells. The nucleic acid sequences of these P-like crassipeptide-encoding clones were determined according to the standard protocol for automated sequencing at the Health Sciences Center Core Sequencing Facility, University of Utah.

Chemical synthesis and oxidative folding of pl14a, cce9a, cce9b and iq19a

The chemical synthesis of κ J-conotoxin pl14a was reported previously (Imperial et al., 2006), as was the chemical synthesis of cce9a (Cabang et al., 2011). The chemical synthesis of P-like crassipeptides cce9b and iq19a was performed using methods described previously (Cabang et al., 2011). Briefly, both peptides were synthesized on an Apex 396 automated peptide synthesizer (AAPPTec, Louisville, KY, USA) using a standard solid-phase Fmoc (9-fluorenylmethyloxycarbonyl) protocol. The peptides were constructed on preloaded Fmoc-L-Val-Wang resin (substitution: 0.53 mmol g⁻¹, Peptides International Inc, KY, USA). The peptides were removed from the resin by treatment with reagent K (TFA/water/phenol/thioanisole/ethanedithiol 82.5/5/5/5/2.5 by volume) for 5 h (cce9b) and 4 h (iq19a), subsequently filtered, precipitated and washed twice with cold methyl-tert-butyl ether. The

crude peptides were dissolved in 10% of solvent B and purified by RP-HPLC using a semi-preparative C₁₈ Vydac column (218TP510, 250 mm × 10 mm, 5 m particle size) with a flow rate of 4 ml/min and a linear gradient ranging from 15% to 45% of solvent B in 30 min for cce9b and 20% to 50% of solvent B in 30 min for iqi9a. Solvents A and B were 0.1% (v/v) TFA in water and 0.1% TFA (v/v) in 90% aqueous acetonitrile, respectively. The absorbance of the eluate was monitored at 220/280 nm. The purity of peptides was assessed using an analytical C₁₈ Vydac reversed-phase HPLC column (218TP54, 250 mm × 4.6 mm, 5 m particle size) with a flow rate of 1 ml/min and a linear gradient ranging from 15% to 45% of solvent B in 30 min for cce9b and 20% to 50% of solvent B in 30 min for iqi9a.

Oxidative folding of cce9b and iqi9a was performed as follows: the linear peptide was re-suspended in a 0.01% TFA solution and added to a solution containing: 0.1 M Tris-HCl (pH 7.5), 0.1 mM EDTA, 1 mM GSH and 1 mM GSSG. The final peptide concentration in the folding mixture was 20 M. The folding reaction was allowed to proceed for 64 min for cce9b and 24h for iqi9a, and then quenched by adding formic acid to a final concentration of 8%. The reaction mixture was separated by RP-HPLC on C₁₈ columns with a linear gradient ranging from 15% to 45% and from 20% to 50% of solvent B in 30 min for cce9b and iqi9a respectively. The purity of the folded peptides was assessed using the method described for the linear peptides and was determined to be: 99% for cce9b and 96% for iqi9a. Both peptides were quantified by absorbance at 280 nm using an extinction coefficient (ϵ) value of 6990 M⁻¹cm⁻¹. Molecular mass was confirmed by ESI MS for cce9b: actual [MH⁺] = 3467.544, calculated [MH⁺] = 3467.487. Molecular mass was confirmed by MALDI MS for iqi9a: actual [MH⁺] = 3223.081, calculated [MH⁺] = 3223.299. The mass spectrometry data was collected at the Mass Spectrometry Core at the Salk Institute for Biological Studies in La Jolla, CA.

Constellation Pharmacology

The Constellation Pharmacology methods have been described in detail previously (Smith et al., 2013; Teichert et al., 2014a; Teichert et al., 2012a; Teichert et al., 2012b). Briefly, lumbar DRG neurons from a postnatal-day 22 mouse (C57BL/6) were removed, pooled and cells were dissociated by enzymatic and mechanical methods, after which the cells were cultured overnight. Cells were loaded with Fura-2-AM dye for calcium imaging. Responses were elicited from neurons by depolarizing the cells at regular time intervals with 25 mM extracellular potassium. Capsaicin (300 nM) was applied at the end of the experiment to identify neurons that express the TRPV1 channel (putative nociceptors). The crassipeptide cce9a (10 M) or the conopeptide pl14a (16 M) were applied to the cell culture while monitoring cytosolic calcium concentration by calcium imaging to determine whether these peptides directly elicited changes in cytosolic calcium concentration or altered the response to a depolarizing stimulus.

Assessment of *in vivo* effects

Each peptide sample was re-suspended in 12 l of saline solution. Using an insulin syringe the samples (5-10nmoles) were injected intracranially to mice that were 12-14 days old. The peptide-injected mice were observed side by side with saline-injected controls for at least 2 h. In these experiments, lethargy was assayed by pushing the mouse a few inches off its

resting position. The saline-injected mouse would go a few to several steps from where it lands; whereas, a lethargic mouse would stay put or move just a step or two from where it lands.

Electrophysiological recordings

The *Xenopus* expression system was used for investigating the potential effects of cce9a, cce9b and iqi9a on voltage-gated *Shaker* K⁺ channels and human Kv1 channels (hKv1.1 - hKv1.7). Oocytes from *Xenopus laevis* were prepared as described previously (Jacobsen et al., 2000). Frogs were anaesthetized with 0.2% tricaine in ice water for surgery. cRNA coding for *Shaker*-K427D and hKv1 channels was injected and the oocytes were incubated 1-5 days to allow expression of the protein. Prior to the electrophysiological measurements, the vitelline membranes of the oocytes were removed mechanically with fine forceps. cRNAs encoding K⁺ channels to be tested were prepared by standard methods as described in (Jacobsen et al., 2000). Whole cell currents were recorded under two-electrode voltage clamp control using a Turbo-Tec amplifier (npi electronic, Tamm Germany). The intracellular electrodes were filled with 2 M KCl and had a resistance between 0.5 and 1.2 MΩ. Current records were low-pass filtered at 1 kHz (-3dB) and sampled at 4 kHz. Leak and capacitive currents were corrected online by using a P/n method. The bath solution was normal frog Ringer's (NFR) containing (in mM): 115 NaCl, 2.5 KCl, 1.8 CaCl₂, 10 Hepes pH 7.2 (NaOH). Lyophilized peptides were dissolved in NFR, diluted to the final concentration and added to the bath chamber. All electrophysiological experiments were performed at room temperature (19-22 °C).

RESULTS

Biology and phylogenetics of crassispirine snails

There is little in the scientific literature that sheds light on the biology of the crassispirine snails; however, as shown in Figure 1, we have recorded observations of envenomation of worm prey by *Crassispira cerithina*, the crassispirine species that is the main focus of this study. The snail extends a pure-white proboscis when it approaches potential prey, and then injects a bolus of venom. Figure 1 was derived from a video taken by Dylan Taylor, which is the first record of envenomation by a crassispirine snail.

The initial member of the crassipeptide family defined in this study was initially discovered through the analysis of the venom ducts from *Crassispira cerithina* (Cabang et al., 2011). This was a peptide called cce9a. In this study, we report the discovery of cce9a homologs from both *Crassispira cerithina* and *Inquisitor intertincta*. These species belong to a monophyletic clade within the family Pseudomelatomidae that largely corresponds to the traditional subfamily Crassispirinae (Figure 2). The phylogenetic tree shows the relationships between these and other crassispirine species, using species from the closely related conoidean families, Drillidae and Clavatulidae, as outgroups. The tree is based on sequences from mitochondrial genes: 12SrRNA, 16SrRNA and COI. It strongly supports the close evolutionary relationship of *Crassispira cerithina* and *Inquisitor intertincta*.

Molecular cloning and synthesis of P-like crassipeptide sequences

Only one venom peptide has been reported from any species in the subfamily Crassispirinae, cce9a from *Crassispira cerithina* (Cabang et al., 2011). This 29 amino-acid peptide was purified from venom by following the bioactivity elicited when venom fractions and subfractions were injected into the mouse CNS. A partial sequence from a cDNA clone was previously obtained. Using this information, additional cDNA clones were analyzed from the venom ducts of *Crassispira cerithina* and *Inquisitor intertincta* as described in Materials and Methods. The results of the cDNA cloning revealed a set of peptides that are clear homologs, as shown in Table 1. The precursor sequences include a clearly defined signal sequence, an intervening propeptide sequence and a mature-peptide sequence at the C-terminus. The predicted mature-peptide sequences obtained from the cDNA clones are shown in Table 2. The amino-acid sequence of cce9a was also determined independently, using peptide purified from venom as previously reported (Cabang et al., 2011).

The sequences are consistent with what would be expected for members of the same venom-peptide superfamily in *Conus*; all of the peptides have a conserved arrangement of cysteine residues in their primary sequence (cysteine framework). This is very similar to the P-conotoxin family from *Conus* (Lirazan et al., 2000; Miles et al., 2002), where the cysteine framework of these peptides is designated as framework IX. We refer to this venom-peptide superfamily from crassispirines as the P-like crassipeptide superfamily. Three of the peptides shown in Table 2 (cce9a, cce9b and iq19a) were chemically synthesized as described in Materials and Methods.

Evaluating the biological activity of P-like crassipeptides using Constellation Pharmacology

The synthetic crassipeptides (cce9a, cce9b and iq19a) were tested for biological activity using the Constellation Pharmacology platform that was previously described (Teichert et al., 2014a; Teichert et al., 2014b; Teichert et al., 2012a; Teichert et al., 2012b). All three peptides caused excitatory effects in a subset of cultured lumbar dorsal root ganglion (DRG) neurons. Figure 3 demonstrates the effects of cce9a and cce9b in dissociated lumbar DRG neurons from a 22-day-old mouse. Notably, each calcium-imaging trace shown in Figure 3 represents the response of a different DRG neuron, from an experiment where the responses of more than 100 neurons were monitored simultaneously (selected traces are shown). The effects of cce9a were compared to another excitatory peptide, κ J-conotoxin pl14a (pl14a), from *Conus planorbis*, which is a blocker of the voltage-gated K channel, $K_v1.6$ (Imperial et al., 2006). The traces in Figure 3 demonstrate that cce9a specifically affected a subset of DRG neurons that were affected by pl14a. Although pl14a affected a relatively broader subset of DRG neurons, including large-diameter capsaicin-resistant neurons, the cce9a effects were limited predominantly to a subset of the small-diameter, capsaicin-sensitive DRG neurons (Figure 3) affected by pl14a.

In the experiments shown in figure 3, greater than 80% of the neurons in culture did not respond to either pl14a (the conopeptide) or to cce9a or cce9b (the crassipeptides). All of the cells that responded to the cce9a peptide (the lowest 6 traces) also responded to the pl14a peptide. However, there were a subset of cells (2nd to 4th traces from the top) which did not

respond to the cce9a peptide, but did respond to the pl14a peptide. Thus, the constellation pharmacology experiment shows that the cce9a peptide affected only a minority of DRG neurons, and all of the neurons affected were also affected by the pl14a peptide. However, the converse was not true; a subset of neurons were affected by pl14a, but not by cce9a. The effects elicited by cce9a and pl14a differed from one neuron to another, as illustrated in Figure 3, but included direct increases in cytosolic calcium concentration as well as amplification of responses to depolarizing stimuli. The conopeptide and the crassipeptide elicited the same types of effects in neurons that were affected by both. These effects are consistent with a variety of other K-channel blockers we have tested previously by the same method (Teichert et al., 2014a; Teichert et al., 2014b; Teichert et al., 2012a; Teichert et al., 2012b).

Using DRG neurons, the effects of cce9a, Iqi9a and cce9b were indistinguishable; the same subset of neurons were affected. When added sequentially, cce9a and cce9b elicited the same phenotypic response in the affected DRG neurons (see Figure 4 for specific examples).

***In vivo* effects; Electrophysiology of cce9b peptide**

The results described above suggest that cce9a and cce9b may have identical molecular targets in mouse DRG cells, overlapping with the molecular targets of the pl14a *Conus* peptide. In order to evaluate the three peptides, cce9a, cce9b and iqi9a further, the synthetic peptides were injected into mice at doses between 5-10 nmoles. Both the cce9a peptide and the iqi9a peptide induced lethargy upon IC injection (see Methods). However, the cce9b peptide had strikingly different symptomatology. Increased excitability was observed — some of the mice exhibited barrel rolling or circling, while other mice had severely splayed legs. All of the mice were generally hypersensitive to touch. Thus, in the 12-14 day old mice, very different behavioral symptomatology was observed with cce9a and cce9b, suggesting differences in targeting selectivity of these peptides. As shown in Table II, cce9b is more divergent in sequence from cce9a (iqi9a differs by two amino acid substitutions, while cce9b has 4 amino acid differences, all of which introduce positive charges). We therefore carried out an electrophysiological characterization of cce9a and cce9b in an effort to further define the divergence in molecular targeting specificity between the two peptides.

The calcium-imaging data strongly indicated that the crassipeptides might be K-channel blockers. We tested these peptides, by electrophysiology on the *Drosophila* Shaker and on human Kv1 channels (see Methods). This *Drosophila* channel has been used as a screen for K channel antagonists since it is generally more permissive in binding K channel antagonists compared to individual mammalian K channel subtypes. An inhibitory activity of the cce9b peptides on the *Drosophila* channel, with weaker activity on hKv1.1 was observed. Application of cce9b caused an inhibition of the current elicited by depolarization of the *Drosophila* Shaker-K427D and hKv1.1 channel (see Figure 5) with IC₅₀ of $1.1 \pm 0.2 \mu\text{M}$ and $2.9 \pm 0.3 \mu\text{M}$ (mean $\pm$ S.E.M.; n=3) respectively. Other hKv1 channels tested (hKv1.2, hKv1.3, hKv1.4, hKv1.5, hKv1.6 and hKv1.7) were not affected by $1 \mu\text{M}$ cce9b (data not shown). These results establish that the cce9b peptide is a selective K-channel blocker. Both the cce9a and the iqi9a peptides (1 M) did not elicit any detectable effects when tested on these targets.

DISCUSSION

P-like crassipeptide superfamily

In this paper, we report the discovery of the first crassipeptide superfamily (P-like crassipeptides, Tables 1 and 2). We have demonstrated that multiple P-like crassipeptides can be expressed in the venom of a single crassispirine species (e.g. *Crassispira cerithina*) and that there are homologous P-like crassipeptides (Tables 1 and 2) found in different species of the former subfamily Crassispirinae (*Crassispira cerithina* and *Inquisitor intertincta*, Figure 2). The definition of the P-like crassipeptide superfamily demonstrates that the venom components in the crassispirine snails are generally similar to the venom peptides of *Conus*. The precursor organization is the same as in *Conus* peptides (Table 1) and the final gene product expressed in the venom is biochemically similar to P-conotoxins, which each have approximately 25 - 30 amino acids and three disulfide bonds (Table 2). The definition of the P-like crassipeptide superfamily and the functional characterization of a few of these peptides are the first steps toward the systematic exploration of this potentially rich pharmacological resource.

A few P-like crassipeptides were synthesized. The bioactivity of the synthetic peptides was first assessed using a platform that we have developed to facilitate discovery called Constellation Pharmacology. In the specific iteration used for these studies, the neuronal preparation used were dorsal root ganglion neurons in primary culture. The specific protocol applied, in which cells are loaded with Fura2 to assess changes in cytosolic calcium, and then pulsed with a KCl solution to induce membrane depolarization in all of the cells in the culture, leads to the simultaneous assessment of many different voltage-gated ion channels. Each dorsal root ganglion is believed to have ca. 25-30 different neuronal subclasses; the amount of calcium entry into the cytosol in a particular neuron will be a function of which isoforms of sodium channels, calcium channels and potassium channels expressed in that particular neuronal subclass. Thus, the protocols shown in Figure 3, allows 100-200 cells to be assayed simultaneously, with the activity of a peptide on all of the voltage-gated sodium, calcium and potassium channels simultaneously assessed. The advantage gained in using this assay is that because it is a high-content, phenotypic assay, if a particular venom peptide is selectively targeted to a specific channel isoform, only those neurons that express that channel isoform will be affected when the peptide is applied. The crassipeptides cce9a and cce9b exhibited similar effects in the same DRG neurons. It is not surprising that cce9a, from the venom of *Crassispira cerithina*, and iqi9a, from the venom of *Inquisitor intertincta*, exhibited similar effects, since these peptides only differ in two amino acids (Tables 1 and 2). Thus, two different species of crassispirine snails express in their venoms homologous P-like crassipeptides that presumably share the same native molecular targets in their prey (although there is no direct information available regarding the specific prey of *Inquisitor intertincta*).

We also found that cce9a and cce9b elicited effects that overlapped with the effects elicited by pl14a (Figure 3), a known blocker of a voltage-gated K channel, Kv1.6 (Imperial et al., 2006). It is notable that the two crassipeptides generally elicited the same specific cellular phenotypes as pl14a in the neurons affected. Since the pl14a peptide causes diverse

phenotypic responses in different DRG neuronal subclasses (see Figure 3), the correspondence in phenotypes suggests that in the neurons affected by pl14a and the crassipeptide, the molecular targets are identical. Notably, the majority of DRG neurons that did not respond to pl14a also were unresponsive to the crassipeptides. However, there was a subset of large DRG neurons that were affected by the pl14a conopeptide that were not affected by cce9a or cce9b (see Figure 3). These results suggest that pl14a has at least two molecular targets, only one of which is targeted by the crassipeptides. Additionally, cce9b blocked potassium currents mediated by the *Drosophila* Shaker K channel and the human K_v1.1 channel (Figure 4).

κP-crassipeptide family

Since the crassispine snails are a large and diverse group, our results suggest that crassispine venoms may become a rich source of K-channel-targeted ligands with novel targeting selectivity. As cce9a, cce9b and iqi9a appear to represent the first members of a novel crassipeptide family of K-channel inhibitors, we suggest adopting a nomenclature parallel to that for conopeptides. Thus, we name cce9a as κP-crassipeptide CceIXA, (where IX is the Roman numeral for “9”), with the peptide name abbreviated as κP_c-CceIXa. Since peptide names are often abbreviated, the family symbol for all crassipeptides for which a mechanism has been elucidated should be abbreviated with the subscript C, indicating that it is a crassipeptide. Similarly, the iqi9a and cce9b peptides will be designated κP-crassipeptide IqiIXA (κP_c-IqiIXA) and κP-crassipeptide CceIXB (κP_c-CceIXB), respectively.

Although we have defined the κP-crassipeptide family, it cannot be assumed that the uncharacterized members of the P-like crassipeptide superfamily (e.g. cce9c and iqi9b) are necessarily K-channel blockers. The sequences are significantly divergent (Table 2), raising the possibility that these peptides do not target K channels. The situation is analogous to that found in *Conus* venoms. One of the best-characterized groups of conotoxins is the O-superfamily, which includes ω-conotoxins that block Ca channels (Yoshikami et al., 1989), and κ-conotoxins that block K channels (Terlau et al., 1996). Similarly, some members of the P-like crassipeptide superfamily may not necessarily be K-channel blockers, and those that do not target K channels would not belong to the κP-crassipeptide family, although if cce9c and iqi9b target K channels, they would be assigned to the κP-crassipeptide family.

It is notable that although they are biochemically similar to conopeptides, the κP-crassipeptides are distinct from the diverse families of K-channel blockers characterized from *Conus* venoms so far. These include the κ-conotoxins (Terlau et al., 1996) that belong to the O-conopeptide superfamily, the κM-conotoxins (Chen et al., 2010) that belong to the M-conopeptide superfamily and the κJ-conotoxins (Imperial et al., 2006) that belong to the J-conopeptide superfamily. Although all of these have 2-3 disulfide bonds, they do not have the same cysteine pattern as the κP-crassipeptides. Thus, the putative K-channel blocking toxins from the crassispine snails comprise a distinctive group of K-channel antagonists that diverge in structure and in their genetic origins from all of the diverse K-channel blocking peptides characterized from *Conus* venoms.

While cce9a, cce9b and iqi9a are analogous functionally to the various families of κ -conotoxins (κ -, κ M- and κ J-conotoxin families), they have greater sequence similarity to the spasmodic peptides from molluscivorous cone snails that define the P-conotoxin superfamily (Lirazán et al., 2000; Miles et al., 2002). As shown in Table 2, there is a striking similarity in the spacing of cysteine residues in the two classes of peptides. This suggests that there may be structural similarity between these peptides, and that the P-like crassipeptides may fold into the ICK motif (inhibitory cysteine knot motif) that has been established for the P-conotoxin superfamily peptides (Miles et al., 2002).

This characterization of the P-like crassipeptides introduces a new paradigm for the discovery and characterization of novel pharmacologically active venom components. The discovery of bioactive compounds from the venoms of crassispine snails presents special challenges compared to the traditional methods used for cone-snail venoms or milked-venom samples from other species. This is because many venomous crassispine species are relatively small and often rare or difficult to collect. Therefore only a very limited amount of biological material is typically available. Although these limitations would have been impenetrable barriers to discovery just a few years ago, advances in molecular biology enable us to obtain venom-peptide sequences from even miniscule amounts of fresh tissue. Many of the crassispine snails are between 5mm-2cm in length, but a single venom duct is now sufficient to obtain a rich harvest of venom-peptide sequences. Thus, the advances in molecular biology make it increasingly easy to obtain venom-peptide sequences. Furthermore, if the encoded venom peptides are under 40 amino acids in length, the chemical synthesis of the predicted peptides, while requiring specialized expertise, can be achieved successfully in most cases. Therefore, the on-going problems in venom-peptide discovery are generally not sequence discovery or peptide synthesis. Rather, the greater problems include how to prioritize which peptides are likely to have the most novel target selectivity and then to identify the targeting-selectivity profiles.

Target identification of bioactive venom peptides that elicit behavioral symptomatology can be particularly problematic. The elucidation of the mechanism that underlies the phenotype observed upon injection into animals can often take years. For example, it was previously known that cce9a was bioactive, since it caused a distinctive behavioral symptomatology: when injected into the central nervous system of adult mice, the peptide induced hyperactivity (Cabang et al., 2011). However, for cce9a, like many other venom peptides, the rate-limiting step has been the evaluation of the peptide's molecular targeting selectivity profile.

Constellation pharmacology

We are developing a form of high-content cell-based phenotypic screening assays that we refer to as “Constellation Pharmacology”. The content of these high content assays is coupled to our on-going work to identify neuronal cell types and the cell-specific combinations of receptors and ion channels that they express (Smith et al., 2013; Teichert et al., 2014a; Teichert et al., 2014b; Teichert et al., 2012a; Teichert et al., 2012b). We refer to such cell-specific combinations of receptors and ion channels as “constellations” and

therefore the pharmacological approach to elucidate these cell-specific constellations as “constellation pharmacology”.

We apply our knowledge of (and pharmacological markers for) cell-specific constellations to help identify the molecular targets of novel pharmacological agents, when we use native heterogeneous cell populations for high-content screening. Currently, we are using calcium-imaging for constellation pharmacology because we have successfully developed assays to interrogate a broad spectrum of receptors and ion channels by calcium imaging. However, we do not intend to use the term “constellation pharmacology” as a synonym for calcium imaging. Constellation pharmacology could potentially also be accomplished with high-throughput electrophysiology, imaging with voltage-sensitive dyes and other technologies. Through the on-going development of constellation pharmacology, we can assay a wide array of receptors and ion channels simultaneously across a heterogeneous cell population. As we apply more pharmacological markers to distinguish between cell types, we have a direct pathway for assessing whether a newly-discovered venom peptide differs in its molecular targeting specificity from already known pharmacological agents.

In this work, we used capsaicin as markers and the conopeptide p114a, a K_v1.6 blocker to identify the neuronal subclasses affected by cce9a. We demonstrated that cce9a elicited an excitatory phenotype in a subset of the small-diameter capsaicin-sensitive DRG neurons that were also affected by p114a (Figure 3). It is notable that the calcium-imaging approach used to assess bioactivity of cce9a also delimits its potential targeting selectivity compared to p114a. Knowing which cells responded to cce9a, and which cells did not, provides a potential path to further identify the K-channel isoforms that are high-affinity targets for this peptide. The fact that p114a affected a broader subset of DRG neurons suggests the possibility that cce9a may be more selective for certain heteromeric K-channel subtypes than p114a, a hypothesis which will be tested in subsequent studies. As we progressively elucidate the cell-specific constellations of each cell type in a heterogeneous cell population, such target discovery will be greatly facilitated.

It is also noteworthy that other cell populations, beyond DRG neurons, can be used for constellation pharmacology, which could also help to narrow the set of potential molecular targets (Raghuraman et al., 2014). Finally, we also note that although the purification of venom peptides was not reported in this work, the same approach can also be used for the bioassay-guided purification of venom components or other complex biological mixtures, as has been demonstrated in a companion study to this work (Aman et al., Manuscript in preparation).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by a program project grant from the National Institute of General Medical Sciences (GM048677), by the Pharmaseas grant from the Republic of the Philippines (to GPC) and from the NIH/NIGMS Fogarty International Center's International Cooperative Biodiversity Groups (ICBG) grant (1U01TW008163). We

are grateful to Dylan Taylor for the videos of the behavior of *Crassispira cerithina*, from which the photographs shown in Figure 1 were derived.

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Highlights for crassipeptide paper

- Venom peptides from marine molluscs now extend from conotoxins to crassipeptides.
- This paper defines the first crassipeptide superfamily, the P-like crassipeptides.
- Three P-like crassipeptides were synthesized and characterized.
- The bioactivity of the characterized peptides suggests they are K channel blockers.

Figure 1.

left panels: *Crassispira cerithina* attacking a worm. Top, the snail has its siphon extended; the snails use a chemosensory mechanism to locate their prey. Middle, the mouth parts of the snail are visible under the siphon, with the white tip of the proboscis (red arrows) extended out. Bottom panel: The extended white proboscis is clearly visible as the snail is ready to strike and inject its venom; the eye stalks of snails are also visible. Right panel. Representatives of the two other genera that group together with *Crassispira cerithina* in a monophyletic clade. Top, *Ptychobela lavina*; bottom, *Inquisitor intertincta*.

Figure 2.

The maximum likelihood tree showing the relationships of *Crassispira cerithina* and *Inquisitor intertincta* (red arrows) to other species in the Crassispirinae, a subfamily of the family Pseudomelatomidae (Bouchet et al., 2011) and to species in two other Conoidean families, Clavatulidae and Drilliidae. Branches are labeled with Bayesian posterior probabilities (left) and approximate Likelihood Ratio (aLRT) statistics (right). The taxonomic assignments of species in the tree (*Crassispira*, *Ptychobela* and *Inquisitor*) are not consistent with the molecular phylogeny, and may need revision in the future once molecular data are obtained from more species.

Figure 3.

The effects of cce9a and pl14a on a subset of DRG neurons. Selected calcium-imaging traces from dissociated DRG neurons show the diverse effects of a P-like crassipeptide, cce9a, and a conopeptide, κ J-conotoxin pl14a, a known blocker of a voltage-gated K channel, $K_V1.6$. Each arrow represents a 15-second application of either 25 mM extracellular potassium (K), to depolarize the neurons, or 300 nM capsaicin (C). Horizontal bars indicate when either cce9a (10 μ M) or pl14a (16 μ M) were present in the bath. (top trace) Responses from a large-diameter capsaicin-resistant neuron that was unaffected by

either cce9a or pl14a. (2nd trace from top) Responses from a large-diameter capsaicin-resistant neuron that was unaffected by cce9a. In contrast, pl14a elicited an increase in the baseline cytosolic calcium concentration that reversed after washout of the peptide. (3rd and 4th traces from top) Responses from two small-diameter capsaicin-sensitive neurons that were unaffected by cce9a. In contrast, pl14a elicited reversible increases in baseline cytosolic calcium concentration, and an amplification of the response to a depolarizing stimulus (K), in the case of the 4th trace. (bottom six traces) Responses from neurons that were affected by both cce9a and pl14a, typically with amplified responses to depolarization (K), but also in some cases with increases in baseline cytosolic calcium concentration (e.g. bottom trace). Notably, all of these neurons were small-diameter capsaicin-sensitive neurons. In a wide variety of DRG neurons, pl14a elicited or amplified responses, whereas cce9a only elicited or amplified responses in a subset of the small-diameter capsaicin-sensitive DRG neurons.

Figure 4.

The crassipeptides cce9a and cce9b amplified responses to a depolarizing stimulus or increased baseline Ca^{2+} concentration in largely the same DRG neurons (small-diameter neurons only). These effects were readily reversible. Arrows indicate brief applications of 20 mM K^+ to depolarize the neurons. Each peptide was applied to the dissociated cell culture at 10 M concentration, as indicated by horizontal bars.

Figure 5.

The crassipeptide cce9b blocks *Shaker*- and hKv1.1-mediated currents. Whole cell currents recorded from an oocyte expressing *Shaker-K427D* (A) and an oocyte expressing hKv1.1 K⁺ channels (B) evoked by test potentials to 10 mV. Addition of 1 μ M of cce9b for *Shaker-K427D* and 3 μ M for hKv1.1 results in a profound block of the currents. The dashed line corresponds to zero current. In contrast, addition of 1 μ M of cce9a and Iqi9a had no effect (results not shown).

Table 1
Comparison of precursor sequences encoding P-like crassipeptides and P-conotoxins

Cloned P-like crassipeptide precursor sequences	
Cce9.1	MKLLPVLVTVVILLQLFADNHARVDGPRAVASGRYATKKAFLQMMTRGSCGLPCH-ENRRCGWACYCDDGICKPLRV*
IqI9.1	MKLLPVLVTVVILLQLFADNHARVDGPRAVASGRYATEKDFLQMMTRGSCGPPCH-ENRRCGWACYCDDGFCKPLRV*
IqI9.3	MKLLPVLVTVVILQLFADNHARVAGPRPVASGQYATEKAFLQMIKRDVCSGSCY-YHYQCSRSCYCHYSHCRDKYEK*
Cce9 . 9	MKLLPVLVTVVILLQLFADNHARVDGPRAVANGRYATEKAFLQMMTRRFCGQSCHGQPSLCHWTCPCNGHFCSRL*
Cloned P-conotoxin precursor sequences	
tx9a	MHLSLARS AVLMLLLFALGNFVVVQSGQITRDVDNGQLTDNRRNLQSKWKPVSLYMSRRGCNNSCQEHSDCESHCICTFRGCGAVNG*
gm9a	MHLSLARS AVLMLLLFALGNFVVVQSLITRDVDNGQLTDNRRNLQTEWNPLSLFMSRRSCNNSCQSHSDCASHCICTFRGCGAVNG*

The signal sequence is in blue. The propeptide sequence is in green. The mature-peptide sequence is in black. Stop codons are indicated by asterisks*.

Table 2
Comparison of mature-peptide sequences from P-like crassipeptides and P-conotoxins

Preliminary peptide name	Amino Acid Sequence	Peptide name following functional characterization
P-like crassipeptide sequences		
cce9a	GSCGLPC ^u HEN-RRCGWACYCDDGICKPLRV	kP-crassipeptide CceIXA
iqi9a	GSCGPPCHEN-RRCGWACYCDDGFCKPLRV	kP-crassipeptide IqiIXA
cce9b	<u>H</u> SCRRHCHEN-RRCGWACYCDDGICKPLRV	kP-crassipeptide CceIXB
iqi9b	DVCSGSCYYH-YQCSRSCYCHYSHCRDKYEK	
cce9c	RFCGQSCHGQPSLCHWTCPCNGHFC SRL	
Alignment of P-conotoxin and P-like crassipeptide sequences		
tx9a (conotoxin)	GCNNSCQ□HSDC□SHCICTFRGCGAVN-NH ₂	
gm9a (conotoxin)	SCNNSCQSHSDCASHCICTFRGCGAVN-NH ₂	
cce9a	GACGLPC ^u HENRRRCGWACYCDDGICKPLRV	
iqi9b	DVCSGSCYYHYQCSRSCYCHYSHCRDKYEK	

Underlined amino acids indicate differences between cce9a, iqi9a and cce9b. Dashes are for alignment of amino acids. The cloned sequences shown in Table 1 encode some of the mature-peptide sequences shown here: Cce9.1 = cce9a, Iqi9.1 = iqi9a, Iqi9.3 = iqi9b, Cce9.9 = cce9c.