Two novel sodium channel inhibitors from *Heriæus melloteei* spider venom differentially interacting with mammalian channel's isoforms[☆]

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ABSTRACT

Two new polypeptide toxins named Hm-1 and Hm-2 were isolated from the venom of the crab spider *Heriæus melloteei*. These toxins consist of 37 and 40 amino acid residues, respectively, contain three intramolecular disulfide bonds, and presumably adopt the inhibitor cystine knot motif. Hm-1 is C-terminally amidated and shows a low degree of homology to spider toxins agelenin and μ -agatoxin-II, whereas Hm-2 has no relevantly related peptide sequences. Hm-1 and Hm-2 were found to act on mammalian voltage-gated Na⁺ channels. Both toxins caused a strong decrease of Na⁺ current peak amplitude, with IC₅₀ values of 336.4 and 154.8 nM, respectively, on Nav1.4. Hm-1 and Hm-2 did not shift the voltage-dependence of activation, nor did they change the kinetics of fast inactivation of the Na⁺ currents. Interestingly, both toxins negatively shifted the steady-state inactivation process, which might have important functional consequences *in vivo*. However, this hyperpolarizing shift cannot by itself explain the observed inhibition of the Na⁺ current, indicating that the two presented toxins could provide important structural information about the interaction of polypeptide inhibitors with voltage-gated Na⁺ channels.

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1. Introduction

Voltage-gated Na⁺ (Nav) channels constitute a large superfamily of membrane-spanning glycoproteins that form ion-conducting pores with a high selectivity for Na⁺ ions. They play a crucial role in processes of eliciting and propagating action potentials in electro-excitable cells as

well as homeostasis maintenance (Catterall, 2000). High-affinity interaction with some specific ligands allows identifying, purifying and characterizing these membrane proteins. Peptides from scorpion, sea anemone, cone snail and spider venoms, in particular, have become a new generation of high-precision tools that serve to investigate ion conductivity, spatial structure, and pharmacological characteristics of Nav channels (Corzo and Escoubas, 2003; Grishin, 1999; Honma and Shiomi, 2006; Rodriguez de la Vega and Possani, 2005; Terlau and Olivera, 2004). In spite of recent success, structure and physiological role of many types of Nav channels are yet unknown. Therefore, finding new specific ligands is an urgent problem that is directed on deciphering molecular aspects of ion channel function.

Nav channels consist of a major pore-forming α -subunit (~260 kDa) and one or more smaller auxiliary β -subunits (30–40 kDa). Expression of the α -subunit alone is sufficient to construct a functional Nav channel, whereas β -subunits modulate the kinetics and voltage-dependence of channel

Abbreviations: HPLC, high-performance liquid chromatography; MALDI, matrix-assisted laser desorption ionization; MS, mass spectrometry; STX, saxitoxin; TFA, trifluoroacetic acid; TTX, tetrodotoxin; Nav channel, voltage-gated sodium channel.

[☆] The protein sequence data reported in this paper will appear in the Uniprot Knowledgebase under the accession numbers P85505 (Hm-1) and P85506 (Hm-2).

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gating and influence channel localization and cell adhesion (Yu and Catterall, 2003). To this moment, nine different mammalian Na_v channel isoforms ($\text{Na}_v1.1$ – $\text{Na}_v1.9$) were cloned, from which the first eight have been functionally expressed. In addition, a 10th related isoform (Na_x) was cloned, which may also act as a Na^+ channel (Catterall et al., 2005). The α -subunit is composed of four homologous domains (DI–DIV), each of which contains six transmembrane segments (S1–S6). In the cell membrane, the first and the fourth domains are located in close proximity to each other, resulting in a clockwise assembly of the four domains which enclose the ion-conducting gateway. The extracellular connecting loop between transmembrane segments S5 and S6 in every domain dips into the membrane and is described to form part of the selectivity pore of the channel. This selectivity pore acts as a filter attributing to the high selectivity of the channel for Na^+ ions. The fourth transmembrane segment (S4) of each domain contains several positively charged amino acid residues and acts as a voltage sensor. Depolarization of the cell membrane causes an outward movement of the voltage sensors, which results in conformational changes of the channel and initiates activation. A short sequence of hydrophobic amino acid residues (Ile-Phe-Met) in the intracellular loop that connects DIII and DIV mediates the fast inactivation of the channel (Catterall, 2000; Denac et al., 2000).

Toxins can exert their actions on Na_v channels through different mechanisms that can be split into two groups: they can inhibit Na^+ conduction of the channel by physically occluding the ion-conducting pore (Na_v channel blockers), or they can alter the channel gating kinetics and voltage-dependence by interacting with a region of the channel that changes conformation during channel gating (gating modifiers). On the α -subunit of mammalian Na_v channels, at least seven ligand-binding receptor sites have been identified using toxins that act on the channel in different manners. Site 1 toxins (tetrodotoxin TTX, saxitoxin STX, and μ -conotoxins) block Na^+ conductance by occluding the channel pore. Most Na_v channel isoforms are reversibly blocked by nanomolar concentrations of STX and TTX, hence they are called tetrodotoxin-sensitive (TTX-S); isoforms $\text{Na}_v1.5$, $\text{Na}_v1.8$ and $\text{Na}_v1.9$ are blocked by significantly higher concentrations of TTX and are referred to as tetrodotoxin-resistant (TTX-R). Site 2 toxins (batrachotoxin, grayanotoxin) cause permanent activation by an inhibition of the channel's fast inactivation and a hyperpolarizing shift in the voltage-dependence of activation; site 3 toxins (α -scorpion toxins, sea anemone toxins and some spider toxins) inhibit fast channel inactivation; site 4 toxins (β -scorpion toxins and some spider toxins) shift voltage-dependence of activation to more hyperpolarizing voltages; site 5 toxins (brevetoxins and ciguatoxins) shift activation and inhibit fast inactivation; site 6 toxins (δ -conotoxins) as well as site 7 toxins (pyrethroids or DDT) cause inhibition of the fast channel inactivation (Cestele and Catterall, 2000; Leipold et al., 2005; Wang and Wang, 2003).

To date, numerous polypeptide Na_v channel gating modifiers have been isolated and characterized from the venom of various animals (Bosmans and Tytgat, 2007a,b; Catterall et al., 2007; Corzo et al., 2007; de la Vega and Possani, 2007; Nicholson et al., 2004; Priest et al., 2007). In contrast,

only a limited number of polypeptide toxins were reported to physically block Na_v channels. Among these, the earlier described μ -conotoxins from cone snails block Na^+ currents in a manner similar to TTX and share an overlapping but not identical binding site with TTX (Li and Tomaselli, 2004). Genuine Na_v channel blockers from spider venoms are represented by just a few examples. These include hainantoxins I and III–V from the spider *Selenocosmia hainana* (Li et al., 2003; Xiao and Liang, 2003a,c), huwentoxins I and IV from *Selenocosmia huwena* (Peng et al., 2002; Wang et al., 2007; Xiao et al., 2008) and Tx 1 from *Phoneutria nigriventer* (Diniz et al., 2006; Martin-Moutot et al., 2006). Most of these spider toxins are thought to interact with receptor site 1 on the Na_v channel and block Na^+ conductance via a mechanism similar to that of TTX and STX.

In this article, we describe the first two Na_v channel inhibitors isolated from the venom of crab spider *Heriades melloteei*, designated as Hm-1 and Hm-2, both active on Na_v channels at concentrations in the 10^2 nM range.

2. Materials and methods

2.1. Toxin purification

Crude venom of the spider *H. melloteei* Simon, 1886 (Araneae, Thomisidae) was purchased from Fauna Laboratories, Ltd. (Republic of Kazakhstan)¹. Lyophilized venom was dissolved in distilled water (10 mg in 1 ml) and separated by reverse-phase high-performance liquid chromatography (HPLC) on a Jupiter C₅ column (4.6 × 150 mm, Phenomenex, USA) using a 60-min linear gradient of acetonitrile (0–60%) in 0.1% (v/v) aqueous trifluoroacetic acid (TFA) at a flow rate of 1 ml/min. At the second stage of separation, the active fractions were exposed to HPLC on a Luna C₈ column (4.6 × 150 mm, Phenomenex) using a 60-min linear gradient (30–70%) of solvent B (50% (v/v) acetonitrile, 20% (v/v) isopropanol and 0.1% TFA) at a flow rate of 1 ml/min. At each stage eluate absorbance was monitored at 280 nm.

2.2. Reduction of disulfide bonds and modification of thiol groups

Purified toxins were dried on a vacuum concentrator and redissolved in 40 μ l of 0.5 M Tris–HCl (pH 8.5), 6 M guanidine hydrochloride and 3 mM EDTA. A 150-fold molar excess of 1,4-dithiothreitol was added to the mixtures. Probes were sealed and incubated at room temperature for 18 h. Alkylation was performed by addition of 4-vinylpyridine solution in 50% (v/v) isopropanol (a threefold excess with respect to dithiothreitol), the reaction was carried out at room temperature for 15 min in the dark. The modified polypeptides were separated by reverse-phase HPLC on a Jupiter C₅ column (2 × 150 mm, Phenomenex) using a 60-min linear gradient of acetonitrile (15–60%) in 0.1% TFA at a flow rate of 0.3 ml/min. Eluate absorbance was monitored at 210 nm.

¹ SPP Code – A125CH1. Could represent a novel species. Classification provided by Andrey Feodorov from Fauna Laboratories, Ltd.

2.3. Protein sequencing

The N-terminal amino acid sequences of the alkylated peptides were determined on a Procise Model 492 protein/peptide sequencer (Applied Biosystems, USA) according to the manufacturer's protocol.

2.4. Endoproteinase Glu-C digestion

Digestion of 1 nmol of pure alkylated peptides was performed with 0.2 µg of endoproteinase Glu-C (Sigma) in 20 µl of 50 mM ammonium bicarbonate buffer (pH 8.0). Probes were incubated for 4 h at 37 °C. Peptide fragments were separated on a Jupiter C₅ column (2 × 150 mm). A 60-min linear gradient of acetonitrile (0–60%) in 0.1% TFA at a flow rate of 0.3 ml/min was used. Eluate absorbance was monitored at 210 nm.

2.5. CNBr cleavage

One nanomole of pure alkylated peptides was dissolved in 20 µl of 80% aqueous TFA and 1 µl of 5 M CNBr in acetonitrile was added. The samples were incubated for 18 h at room temperature in the dark. The reaction was terminated by diluting the mixture with 1 ml of distilled water, and the samples were dried using a vacuum evaporator. Reaction products were separated on a Jupiter C₅ column as described in Section 2.4.

2.6. Mass spectrometry (MS) analysis

The molecular masses of the peptides were measured by matrix-assisted laser desorption ionization (MALDI) mass spectrometry (MS), a MALDI LR (Micromass UK Ltd., UK) instrument was used. Calibration was performed using either a ProteoMass peptide and protein MALDI-MS calibration kit (mass range of 700–66,000 Da) or a ProteoMass peptide MALDI-MS calibration kit (mass range of 700–3500 Da) (both from Sigma). Molecular masses were determined in a linear positive ion mode. 2,5-Dihydroxy benzoic acid (10 mg/ml in 70% (v/v) acetonitrile, 0.1% TFA) matrix was used.

2.7. Expression of voltage-gated sodium channels in *Xenopus laevis* oocytes

For expression in *Xenopus* oocytes, the cDNA encoding rNav_v1.2 and mNav_v1.6 was subcloned into vector pLCT1 and the rNav_v1.4 cDNA was subcloned into vector pUI-2. For *in vitro* transcription, these plasmids were linearized with NotI. The hβ₁/pGEM-HE construct was linearized with NheI. Next, capped cRNAs were synthesized from the linearized plasmid using the T7 mMESSAGE-mACHINE transcription kit (Ambion, USA). The hNav_v1.5/pSP64T, rNav_v1.8/pBSTA and rβ₁/pSP64T plasmids were linearized with XbaI, NotI and EcoRI, respectively, and transcribed with the SP6 mMESSAGE-mACHINE transcription kit (Ambion, USA).

Stage V–VI oocytes were harvested from the ovarian lobes of anaesthetized female *X. laevis* frogs as described previously (Liman et al., 1992). Oocytes were injected

with 50 nl of cRNA (ratio α:β-subunit of 1:1) at a concentration of 1 ng/nl using a micro-injector (Drummond Scientific, USA). The incubation solution for the oocytes contained (in mM): NaCl 96, KCl 2, CaCl₂ 1.8, MgCl₂ 2 and HEPES 5 (pH 7.4) and was supplemented with 50 mg/l gentamycin sulfate.

2.8. Electrophysiological recordings

Currents were recorded using the two-electrode voltage clamp method. The experiments were performed at room temperature (18–22 °C) using a GeneClamp 500 amplifier (Axon Instruments, USA) controlled by a pClamp data acquisition system (Axon Instruments, USA). Whole cell currents from oocytes were recorded 2–5 days after injection. The voltage and current electrodes were filled with 3 M KCl. Resistances of both electrodes were kept as low as possible (<1.0 MΩ). The elicited currents were sampled at 10 kHz and filtered at 1 kHz using a four-pole low-pass Bessel filter. To eliminate the effect of the voltage drop across the bath grounding electrode, the bath potential was actively controlled by a two-electrode bath clamp. Leak subtraction was performed using a -P/4 protocol. The bath solution was composed of (in mM): NaCl 96, KCl 2, CaCl₂ 1.8, MgCl₂ 2 and HEPES 5 (pH 7.4).

To electrophysiologically characterize the toxins Hm-1 and Hm-2, the following voltage protocols were applied. (1) From a holding potential of -90 mV, current traces were elicited by 100-ms depolarizations at a frequency of 0.2 Hz, to the voltage of maximal activation of the Na_v channel isoform in control conditions. (2) To assess the concentration dependency of the toxin-induced inhibitory effects, dose-response curves were constructed for both toxins. In the presence and absence of a range of concentrations, current traces were evoked as described above. The percentage of toxin-induced inhibition of the Na⁺ current peak amplitudes was plotted against the logarithm of applied concentrations and fitted with the Hill equation to obtain the IC₅₀ value (i.e. the toxin concentration which produces 50% of the maximal effect) of the effects of Hm-1 and Hm-2. From the Hill equation, the Hill coefficient (i.e. the number of molecules necessary to block one channel) was obtained. (3) In order to investigate the effects of both toxins on the voltage-dependence of activation, current traces were evoked at 0.2 Hz from a holding potential of -90 mV, by 100-ms depolarizations from -70 to 40 mV with 5-mV increments. The Na⁺ conductances were calculated from the Ohm's law, normalized and plotted as a function of voltage. To obtain an activation curve, this data set was fitted with the Boltzmann equation, yielding the V_g value (i.e. the voltage of half maximal activation). (4) The effects of Hm-1 and Hm-2 on the steady-state inactivation were determined by a standard double-pulse protocol in which, from a holding potential of -90 mV, 100-ms conditioning pulses from -120 to 10 mV with 5-mV increments were applied. The conditioning pulse was followed immediately by a 100-ms test pulse to the voltage of maximal activation of the Na_v channel in control conditions. The repetition interval was 5 s. The peak Na⁺ currents elicited in the test pulse were normalized, plotted against voltage and fitted with the Boltzmann equation, yielding the V_h

value (i.e. the voltage of half maximal steady-state inactivation).

All data were analyzed with Origin software (OriginLab Corp., USA). To determine statistically significant differences between two means, the Student's *t*-test was used with a probability level of 95%. All data are presented as mean $\pm$ standard error.

3. Results

Initial screening of a collection of spider venoms identified the crab spider *H. mellotei* (family Thomisidae)¹ venom to possess a potent blocking activity towards different Na_v channel isoforms. This venom was used to identify novel polypeptide toxins affecting Na_v channels via a bioassay-guided purification.

3.1. Toxin isolation and purification

The new toxins were isolated using a two-step reverse-phase HPLC. In the first step, crude *H. mellotei* venom was fractionated on a Jupiter C₅ column (Fig. 1A). Separation produced several broad peaks that were combined into 10 fractions and tested. The active fractions 6 and 7 were further separated on a Luna C₈ column using a mixture of acetonitrile and isopropanol as the mobile phase (Fig. 1B and C). The active fractions were named Hm-1 and Hm-2 and their 95% purity was verified by analytical chromatography and MALDI-MS. The measured molecular masses of the new peptides were 4171.9 and 4555.3 Da.

3.2. Amino acid sequence analysis

Alkylation of non-reduced molecules with 4-vinylpyridine revealed no free thiol groups in the native peptides as indicated by MS analysis. Complete amino acid sequences of the peptides (see Fig. 2) were identified by a combination of Edman degradation, selective proteolysis and MS. Direct N-terminal sequencing of reduced and alkylated peptides provided 37 and 40 amino acid long sequences for Hm-1 and Hm-2, respectively, both containing six half-cystine residues. To verify the deduced sequences, the reduced-alkylated peptides were hydrolyzed with endoproteinase Glu-C and cyanogen bromide. The measured molecular mass of Hm-1 C-terminal CNBr (TLSCTRNF, 1045.2 Da) and Glu-C (FWSGPWCCAGKCKLVWSMTLSCTRNF, 3546.1 Da) fragments differed from the calculated values (1046.2 and 3547.3 Da, respectively) by 1 Da. It was concluded that Hm-1 is post-translationally modified and has an amidated C-terminal residue. For other peptide fragments the measured molecular masses matched the calculated values. Thus, Hm-1 and Hm-2 were determined as single-chain polypeptides containing 37 and 40 amino acid residues, respectively, including six cysteines involved in three intramolecular disulfide bridges (Fig. 2).

3.3. Effects of Hm-1 and Hm-2 on Na_v channels

Using the two-electrode voltage clamp technique, the effects of Hm-1 and Hm-2 were assessed on cloned Na_v channel isoforms expressed in *Xenopus* oocytes. First,

Fig. 1. Toxin purification. (A) Separation of 20 μl of *H. mellotei* crude venom using reverse-phase HPLC on a Jupiter C₅ column. Second step separation of fractions 6 (B) and 7 (C) on a Luna C₈ column. The active fractions are indicated with arrows.

Hm-1 GCIPYGKTCEFWSGPWCCAGKKLNVWSMTLSCTRNF-NH₂
Hm-2 GCIPSFGECAWFSGESCTGIKWVFFTSKFMCRRVWGKD-OH

Fig. 2. Amino acid sequences of Hm-1 and Hm-2 toxins. Aromatic amino acid residues are underlined, cysteine residues are shaded grey, methionine and glutamic acid residues are in bold.

Hm-1 and Hm-2 were tested on mammalian Na_v channel isoforms Na_v1.2, Na_v1.4–Na_v1.6 and Na_v1.8 (Fig. 4A). At a concentration of 200 nM, both toxins significantly decreased the peak currents of all tested Na_v channel isoforms, except for the TTX-R isoform Na_v1.8 which was not affected ($n = 3–4$). Hm-1 blocked all other tested Na_v channel isoforms with a similar potency: 200 nM Hm-1 reduced the peak amplitudes of Na_v1.2, Na_v1.4–Na_v1.6 by 32.4 ± 12.6 , 40.0 ± 6.0 , 36.5 ± 4.7 and $38.7 \pm 4.6\%$, respectively ($n = 4–12$). Hm-2, on the other hand, appeared to exhibit a slight preference for Na_v1.2 ($64.6 \pm 7.7\%$ of block at 200 nM, $n = 5$) and Na_v1.4 ($61.9 \pm 5.1\%$ of block at 200 nM, $n = 7$), compared to Na_v1.5 and Na_v1.6 (inhibition of peak amplitudes by $17.8 \pm 3.8\%$ ($n = 6$) and $38.7 \pm 8.2\%$ ($n = 3$), respectively). Since the effects of Hm-1 and Hm-2 appeared to be the most pronounced on Na_v1.4, the effects of both toxins on this Na_v channel isoform were investigated more into detail. A dose–response curve was constructed showing the concentration dependency of the inhibitory effects of Hm-1 and Hm-2 on the peak Na⁺ currents of Na_v1.4 (Fig. 4B). Hm-1 was shown to inhibit the Na_v1.4 channel with an IC₅₀ value of 336.4 ± 40.1 nM ($n = 3–12$, Hill coefficient = 1.0 ± 0.1) whereas Hm-2 affects this Na_v channel isoform with an IC₅₀ value of 154.8 ± 11.2 nM ($n = 3–9$, Hill coefficient = 1.2 ± 0.1). Both Hill coefficients suggest a one-to-one toxin-channel interaction.

3.4. Effects of Hm-1 and Hm-2 on the gating of Na_v channels

In order to investigate the mechanism behind the toxin-induced inhibition of Na_v channels by Hm-1 and Hm-2, their effects on the voltage-dependence of activation and steady-state inactivation of rNa_v1.4 were examined. The current–voltage relationships in control conditions and after addition of 200 nM Hm-1 and Hm-2 show that both toxins inhibit the Na⁺ current without changing the reversal potential, indicating that the ion selectivity of the Na_v channel does not change (Fig. 5A). The toxins do not shift the threshold potential of activation or voltage corresponding to maximal current. To quantify the effects of the toxins on Na⁺ channel gating, activation and steady-state inactivation curves were constructed as described above (see Fig. 5B). The steady-state inactivation curves (■ – control; □ – 200 nM toxin) show that both toxins significantly shift

the V_h ($p < 0.05$) towards more hyperpolarized potentials. Hm-1 causes a shift in V_h from -53.7 ± 0.8 to -59.9 ± 1.2 mV ($n = 3$) and Hm-2 shifts the V_h from -47.4 ± 0.5 to -54.4 ± 0.8 mV ($n = 9$). Furthermore, the activation curves (● – control; ○ – 200 nM toxin) show that Hm-1 and Hm-2 cause a small depolarizing but non-significant shift in V_g ($p > 0.05$), from -27.8 ± 0.5 to -25.7 ± 0.3 mV ($n = 3$) and from -22.8 ± 0.7 to -19.3 ± 0.8 mV ($n = 9$), respectively.

4. Discussion

A large number of animals are able to produce venom that contains substances acting on different targets in the nervous system such as ion channels and membrane receptors. Peptide toxins are the most useful and efficient tools used for ion channel studies because of their high affinity and specificity of action. In the present work, we investigated the primary structure and pharmacological function of Hm-1 and Hm-2, the first two toxins isolated from *H. mellotei* spider venom that inhibit Na_v channels.

The amino acid sequences of the new toxins were established by conventional methods of protein chemistry and mass spectrometry. Hm-1 and Hm-2 share a primary sequence identity of 38%. Similarity searches in protein sequence databases revealed that Hm-1 shows a low degree of homology with toxins from spider venoms: agelenin (40% identity) (Hagiwara et al., 1990; Hagiwara et al., 1991) and μ -agatoxin-II (32%) (Skinner et al., 1989) (Fig. 3). Agelenin from the spider *Agelena opulenta* is composed of 35 amino acid residues with three disulfide bonds. This toxin was suggested to irreversibly inhibit invertebrate pre-synaptic calcium channels, but the mechanism of its action is still unknown (Inui et al., 1992). μ -Agatoxin-II from *Agelenopsis aperta* is a 37 amino acid residues-long peptide with four disulfide bonds. Its molecular target is yet unknown, but this toxin is thought to act on Na_v channels (Adams et al., 1989; Omecinsky et al., 1996). In contrast to Hm-1, Hm-2 has no relevantly similar sequences in protein databases. Hm-1 and Hm-2 each contain six highly conserved half-cystines and therefore possibly have the same disulfide-bonding pattern and similar three-dimensional structure resembling the inhibitor cystine knot (ICK) fold common to most spider neurotoxins (Pallaghy et al., 1994). Another remarkable feature of the new toxins is

Hm-1	-GCIPYKGTCEFWSGPWCCAG-KCKLNVWSMTLSCTRNF	37
agelenin	GGCLPHNRFNCNALSGPRCCSGLKCKELSIWDSR-CL---	35
μ -agatoxin-II	-ECATKNKRCADWAGPWCCDGLYCSCRYPGCM-GRPSS	37

Fig. 3. Amino acid sequence alignment of Hm-1 with agelenin and μ -agatoxin-II – toxins from the spiders *Agelena opulenta* and *Agelenopsis aperta*, respectively. The number of amino acid residues in the polypeptide chain of the corresponding toxin is indicated in the right column. Cysteine residues are shaded dark grey. Identical residues are shaded light grey. Alignment of Hm-2 is not shown because of negligible sequence similarity with other known peptides.

Fig. 4. Effects of Hm-1 and Hm-2 on different mammalian Na_V channel isoforms. (A) Representative current traces of cloned Na_V channel isoforms rNa_V1.2, rNa_V1.4, hNa_V1.5, mNa_V1.6 and rNa_V1.8 expressed in *Xenopus* oocytes. The arrow indicates the steady-state Na⁺ current peak amplitude in the presence of 200 nM toxin, with Hm-1 on the left panels and Hm-2 on the right panels. The dotted line indicates the zero-current level. (B) Dose-response curves for Hm-1 (left panel) and Hm-2 (right panel) on rNa_V1.4. Both curves were fitted with the Hill equation, yielding IC₅₀ values of 336.4 ± 40.1 and 154.8 ± 11.2 nM for Hm-1 and Hm-2, respectively.

the high content of aromatic amino acid residues (see Fig. 2). As a result, these residues are proposed to play a special role in the interaction with channel protein via formation of hydrophobic clusters.

At present, the following Na_V channel blockers from spider venoms have been described. The hainantoxins from

S. hainana represent the first discovered family of spider peptides that selectively block Na⁺ conductance: HNTX-I specifically acts on the para/tipE insect Na_V channel (Li et al., 2003), whereas HNTX-III-V block mammalian TTX-S Na_V channels at nanomolar concentrations (Liu et al., 2003; Xiao and Liang, 2003a,b,c). Huwentoxins I and IV from

Fig. 5. Effects of Hm-1 and Hm-2 on the current-voltage relationships and voltage-dependence of steady-state inactivation and activation of rNav1.4. (A) Current-voltage relationships of a representative experiment in control (●) and after the addition (○) of 200 nM Hm-1 (left panel) and 200 nM Hm-2 (right panel). (B) Voltage-dependence of steady-state inactivation and activation in control (■ and ●, respectively) and after the addition (□ and ○, respectively) of 200 nM Hm-1 (left panel) and 200 nM Hm-2 (right panel).

S. huwena inhibit TTX-S Na_V channels in adult rat dorsal root ganglion neurons, also at nanomolar concentrations (Peng et al., 2002; Wang et al., 2007; Xiao et al., 2008). Furthermore, the structurally peculiar *P. nigriventer* toxin 1 (Tx 1) was shown to block Na_V channels with a high sub-nanomolar affinity (Diniz et al., 2006; Martin-Moutot et al., 2006).

In this study, Hm-1 and Hm-2 were found to significantly inhibit cloned Na_V channels expressed in *Xenopus* oocytes. Both toxins significantly decreased Na^+ current peak amplitudes of all tested TTX-S isoforms without significantly altering the fast inactivation kinetics of the Na^+ currents. The TTX-S isoform $\text{Na}_V1.4$ appeared to be one of the most affected isoforms by both toxins, with IC_{50} values of 336.4 ± 40.1 and 154.8 ± 11.2 nM, respectively. The current-voltage relationships (Fig. 5A) show that Hm-1 and Hm-2 did not change the threshold potential of activation or the voltage corresponding to maximal current. Moreover, the reversal potential of the $\text{Na}_V1.4$ currents was not altered, indicating that the toxins did not change the ion selectivity of the Na_V channel. Both Hm-1 and Hm-2 caused a small but significant hyperpolarizing shift of about 6–7 mV in the midpoint of steady-state inactivation (Fig. 5B). Similar observations were made in electrophysiological studies with other spider toxins that inhibit Na^+ channels, such as HNTX-III-V (Xiao and Liang, 2003a,b,c) and HWTX-I (Wang et al., 2007). Altogether, these results show that the mechanism of action of Hm-1 and Hm-2 differs from that of gating modifier toxins that slow the fast inactivation by binding to receptor site 3 (Bosmans and Tytgat, 2007a; Gordon et al., 2007; Nicholson et al., 2004) or shift the voltage-dependence of activation upon binding to receptor site 4 (Bosmans et al., 2007; de la Vega and Possani, 2007) of Na_V channels.

The newly characterized toxins described in this article act on mammalian Na_V channels at relatively high concentrations (10^2 nM range), compared to most of the listed known Na_V channel inhibitors. We therefore speculate that the natural target of Hm-1 and Hm-2 is most probably insect Na^+ channels since insects constitute the natural prey of most spiders including *H. mellotei*. The originality of the presented toxins is in their pharmacological behavior, phenotypically similar to that of classical Na_V channel blockers TTX and STX (Cestele and Catterall, 2000). Therefore, it seems reasonable to assume that Hm-1 and Hm-2 interact with the same site on Na_V channels as TTX and STX, i.e. receptor site 1, although this hypothesis definitely needs to be further supported by isotope-labeled toxin binding assays or mutagenesis studies. However, another equally plausible explanation is that Hm-1 and Hm-2 inhibit Na_V channels by binding to a voltage sensor loop on one of the I–IV pseudo-domains and thereby strongly stabilize the inactivated (i.e. functionally closed) state of the channel, effectively giving rise to reduced channel opening in the normal voltage range. Such a mechanism would be similar to the action of hanatoxin on K_V channels (Phillips et al., 2005; Swartz, 2007). However, in the case of K_V channels, the strongly stabilizing effect on the closed state of the channel results in a decreased transition from the resting state to the open state, whereas in the case of Na_V channels, the toxin promotes the inactivated state as judged from the hyperpolarizing shift seen in the steady-state inactivation curves (see Fig. 5B). Indeed, Hm-1 and Hm-2 do not provoke a higher block at moderate test potentials (e.g. –30 mV), as compared with stronger depolarizations (e.g. –20 mV and more) (see Fig. 5A). In contrast, in the case of a K_V channel, the most pronounced toxin-induced block would be

expected at more moderate depolarizations. Further experiments will be proven useful to elucidate the exact mechanism of action of both toxins.

Negligible sequence similarity to other known polypeptides allows us to propose that both Hm-1 and Hm-2 may belong to a new group of Na_v channel inhibitors. Whether the shift of the steady-state inactivation can be viewed as a hallmark thereof remains to be seen. The newly described toxins are two interesting subjects for future structure–function relationship studies, especially focusing on the large amount of hydrophobic residues. In this way Hm-1 and Hm-2 could provide novel important structural information about the interaction between polypeptide inhibitors and Na_v channels.

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Conflict of interest

The authors of the manuscript entitled: “Two novel sodium channel inhibitors from *Heriæus mellei* spider venom differentially interacting with mammalian channel's isoforms” declare that there are no conflicts of interest associated with this work submitted to Toxicon.

References

Adams, M.E., Herold, E.E., Venema, V.J., 1989. Two classes of channel-specific toxins from funnel web spider venom. *J. Comp. Physiol. A* 164 (3), 333–342.

Bosmans, F., Martin-Eauclaire, M.F., Tytgat, J., 2007. Differential effects of five ‘classical’ scorpion beta-toxins on rNav1.2a and DmNav1 provide clues on species-selectivity. *Toxicol. Appl. Pharmacol.* 218 (1), 45–51.

Bosmans, F., Tytgat, J., 2007a. Sea anemone venom as a source of insecticidal peptides acting on voltage-gated Na⁺ channels. *Toxicon* 49 (4), 550–560.

Bosmans, F., Tytgat, J., 2007b. Voltage-gated sodium channel modulation by scorpion alpha-toxins. *Toxicon* 49 (2), 142–158.

Catterall, W.A., 2000. From ionic currents to molecular mechanisms: the structure and function of voltage-gated sodium channels. *Neuron* 26 (1), 13–25.

Catterall, W.A., Cestele, S., Yarov-Yarovoy, V., Yu, F.H., Konoki, K., Scheuer, T., 2007. Voltage-gated ion channels and gating modifier toxins. *Toxicon* 49 (2), 124–141.

Catterall, W.A., Goldin, A.L., Waxman, S.G., 2005. International Union of Pharmacology. XLVII. Nomenclature and structure–function relationships of voltage-gated sodium channels. *Pharmacol. Rev.* 57 (4), 397–409.

Cestele, S., Catterall, W.A., 2000. Molecular mechanisms of neurotoxin action on voltage-gated sodium channels. *Biochimie* 82 (9–10), 883–892.

Corzo, G., Escoubas, P., 2003. Pharmacologically active spider peptide toxins. *Cell. Mol. Life Sci.* 60 (11), 2409–2426.

Corzo, G., Sabo, J.K., Bosmans, F., Billen, B., Villegas, E., Tytgat, J., Norton, R. S., 2007. Solution structure and alanine scan of a spider toxin that affects the activation of mammalian voltage-gated sodium channels. *J. Biol. Chem.* 282 (7), 4643–4652.

Denac, H., Mevissen, M., Scholtysik, G., 2000. Structure, function and pharmacology of voltage-gated sodium channels. *Naunyn-Schmiedeberg's Arch. Pharmacol.* 362 (6), 453–479.

Diniz, M.R., Theakston, R.D., Crampton, J.M., Nascimento Cordeiro, M., Pimenta, A.M., De Lima, M.E., Diniz, C.R., 2006. Functional expression and purification of recombinant Tx1, a sodium channel blocker neurotoxin from the venom of the Brazilian “armed” spider, *Phoneutria nigriventer*. *Protein Expr. Purif.* 50 (1), 18–24.

Gordon, D., Karbat, I., Ilan, N., Cohen, L., Kahn, R., Gilles, N., Dong, K., Stuhmer, W., Tytgat, J., Gurevitz, M., 2007. The differential preference of scorpion alpha-toxins for insect or mammalian sodium channels: implications for improved insect control. *Toxicon* 49 (4), 452–472.

Grishin, E., 1999. Polypeptide neurotoxins from spider venoms. *Eur. J. Biochem.* 264 (2), 276–280.

Hagiwara, K., Sakai, T., Miwa, A., Kawai, N., Nakajima, T., 1990. Complete amino acid sequence of a new type of neurotoxin from the venom of the spider, *Agelena opulenta*. *Biomed. Res.* 11, 181–186.

Hagiwara, K., Sakai, T., Miwa, A., Kawai, N., Nakajima, T., 1991. Agelenin – a new neurotoxin from the venom of the spider, *Agelena opulenta*. *Pept. Chem.*, 351–356.

Honma, T., Shiomi, K., 2006. Peptide toxins in sea anemones: structural and functional aspects. *Mar. Biotechnol.* 8 (1), 1–10.

Inui, T., Hagiwara, K., Nakajima, K., Kimura, T., Nakajima, T., Sakakibara, S., 1992. Synthesis and disulfide structure determination of agelenin: identification of the carboxy-terminus as an amide form. *Pept. Res.* 5, 140–144.

Leipold, E., Hansel, A., Olivera, B.M., Terlau, H., Heinemann, S.H., 2005. Molecular interaction of delta-conotoxins with voltage-gated sodium channels. *FEBS Lett.* 579 (18), 3881–3884.

Li, D., Xiao, Y., Hu, W., Xie, J., Bosmans, F., Tytgat, J., Liang, S., 2003. Function and solution structure of hainantoxin-I, a novel insect sodium channel inhibitor from the Chinese bird spider *Selenocosmia hainana*. *FEBS Lett.* 555 (3), 616–622.

Li, R.A., Tomaselli, G.F., 2004. Using the deadly mu-conotoxins as probes of voltage-gated sodium channels. *Toxicon* 44 (2), 117–122.

Liman, E.R., Tytgat, J., Hess, P., 1992. Subunit stoichiometry of a mammalian K⁺ channel determined by construction of multimeric cDNAs. *Neuron* 9 (5), 861–871.

Liu, Z., Dai, J., Chen, Z., Hu, W., Xiao, Y., Liang, S., 2003. Isolation and characterization of hainantoxin-IV, a novel antagonist of tetrodotoxin-sensitive sodium channels from the Chinese bird spider *Selenocosmia hainana*. *Cell. Mol. Life Sci.* 60 (5), 972–978.

Martin-Moutot, N., Mansuelle, P., Alcaraz, G., Dos Santos, R.G., Cordeiro, M.N., De Lima, M.E., Seagar, M., Van Renterghem, C., 2006. *Phoneutria nigriventer* toxin 1: a novel, state-dependent inhibitor of neuronal sodium channels that interacts with micro conotoxin binding sites. *Mol. Pharmacol.* 69 (6), 1931–1937.

Nicholson, G.M., Little, M.J., Birinyi-Strachan, L.C., 2004. Structure and function of delta-atracotoxins: lethal neurotoxins targeting the voltage-gated sodium channel. *Toxicon* 43 (5), 587–599.

Omecinsky, D.O., Holub, K.E., Adams, M.E., Reilly, M.D., 1996. Three-dimensional structure analysis of mu-agatoxins: further evidence for common motifs among neurotoxins with diverse ion channel specificities. *Biochemistry* 35 (9), 2836–2844.

Pallaghy, P.K., Nielsen, K.J., Craik, D.J., Norton, R.S., 1994. A common structural motif incorporating a cystine knot and a triple-stranded beta-sheet in toxic and inhibitory polypeptides. *Protein Sci.* 3 (10), 1833–1839.

Peng, K., Shu, Q., Liu, Z., Liang, S., 2002. Function and solution structure of huwentoxin-IV, a potent neuronal tetrodotoxin (TTX)-sensitive sodium channel antagonist from Chinese bird spider *Selenocosmia huwena*. *J. Biol. Chem.* 277 (49), 47564–47571.

Phillips, L.R., Milesu, M., Li-Smerin, Y., Mindell, J.A., Kim, J.I., Swartz, K.J., 2005. Voltage-sensor activation with a tarantula toxin as cargo. *Nature* 436 (7052), 857–860.

Priest, B.T., Blumenthal, K.M., Smith, J.J., Warren, V.A., Smith, M.M., 2007. ProTx-I and ProTx-II: gating modifiers of voltage-gated sodium channels. *Toxicon* 49 (2), 194–201.

- Rodriguez de la Vega, R.C., Possani, L.D., 2005. Overview of scorpion toxins specific for Na⁺ channels and related peptides: biodiversity, structure–function relationships and evolution. *Toxicon* 46 (8), 831–844.
- Skinner, W.S., Adams, M.E., Quistad, G.B., Kataoka, H., Cesarin, B.J., Enderlin, F.E., Schooley, D.A., 1989. Purification and characterization of two classes of neurotoxins from the funnel web spider, *Agelenopsis aperta*. *J. Biol. Chem.* 264 (4), 2150–2155.
- Swartz, K.J., 2007. Tarantula toxins interacting with voltage sensors in potassium channels. *Toxicon* 49 (2), 213–230.
- Terlau, H., Olivera, B.M., 2004. Conus venoms: a rich source of novel ion channel-targeted peptides. *Physiol. Rev.* 84 (1), 41–68.
- de la Vega, R.C., Possani, L.D., 2007. Novel paradigms on scorpion toxins that affects the activating mechanism of sodium channels. *Toxicon* 49 (2), 171–180.
- Wang, M., Guan, X., Liang, S., 2007. The cross channel activities of spider neurotoxin huwentoxin-I on rat dorsal root ganglion neurons. *Biochem. Biophys. Res. Commun.* 357 (3), 579–583.
- Wang, S.Y., Wang, G.K., 2003. Voltage-gated sodium channels as primary targets of diverse lipid-soluble neurotoxins. *Cell. Signal.* 15 (2), 151–159.
- Xiao, Y., Liang, S., 2003a. Inhibition of neuronal tetrodotoxin-sensitive Na⁺ channels by two spider toxins: hainantoxin-III and hainantoxin-IV. *Eur. J. Pharmacol.* 477 (1), 1–7.
- Xiao, Y.C., Liang, S.P., 2003b. Inhibition of sodium channels in rat dorsal root ganglion neurons by hainantoxin-IV, a novel spider toxin. *Sheng Wu Hua Xue Yu Sheng Wu Wu Li Xue Bao* 35 (1), 82–85.
- Xiao, Y.C., Liang, S.P., 2003c. Purification and characterization of hainantoxin-V, a tetrodotoxin-sensitive sodium channel inhibitor from the venom of the spider *Selenocosmia hainana*. *Toxicon* 41 (6), 643–650.
- Xiao, Y., Luo, X., Kuang, F., Deng, M., Wang, M., Zeng, X., Liang, S., 2008. Synthesis and characterization of huwentoxin-IV, a neurotoxin inhibiting central neuronal sodium channels. *Toxicon* 51 (2), 230–239.
- Yu, F.H., Catterall, W.A., 2003. Overview of the voltage-gated sodium channel family. *Genome Biol.* 4 (3), 207.