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Isolation of a Na_v channel blocking polypeptide from *Cyanea capillata* medusae - a neurotoxin contained in fishing tentacle isorhizas.

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Abstract

Jellyfish are efficient predators which prey on crabs, fish larvae, and small fish. Their venoms consist of various toxins including neurotoxins that paralyse prey organisms immediately. One possible mode of action of neurotoxins is the blockage of voltage-gated sodium (Na_v) channels. A novel polypeptide with Na_v channel blocking activity was isolated from the northern Scyphozoa *Cyanea capillata* (L., 1758). For that purpose, a bioactivity-guided multidimensional liquid chromatographic purification method has been developed. A neurotoxic activity of resulting chromatographic fractions was demonstrated by a bioassay, which based on the mouse neuroblastoma cell line Neuro2A. The purification process yielded one fraction containing a single polypeptide with proven activity. The molecular weight of 8.22 kDa was determined by matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-ToF MS). Utilising Laser Microdissection and Pressure Catapulting (LMPC) for the separation of different nematocyst types in combination with direct MALDI-ToF MS analysis of the intact capsules, the neurotoxin was found to be present in all types of fishing tentacle isorhizas (A-isorhizas, a-isorhizas, O-isorhizas) of *C. capillata* medusae.

Keywords: Jellyfish, *Cyanea*, Nematocysts, Neurotoxin, Mass spectrometry

1. Introduction

The toxic effects of the widespread boreal Scyphozoa *Cyanea capillata* (L.) are caused by venomous, basically proteinaceous, compounds (Rice and Powell, 1972). These effects have been investigated in several *in vivo* and *in vitro* studies. Lytic, cytotoxic, enzymatic, and cardiotoxic activities have been demonstrated (Walker, 1977 a,b; Walker et al., 1977; Burnett and Calton, 1987; Long and Burnett, 1989; Helmholz et al., 2007; Helmholz, 2010, Helmholz et al., 2010; Xiao et al., 2010a, b).

A first investigation of *C. capillata* medusae regarding their neurotoxic activity was carried out by Lassen et al. (2010). They showed a blocking activity on voltage-gated sodium (Na_v) channels expressed by mouse neuronal cells in partially purified fishing tentacle venom. Na_v channels play an essential role in the initiation and propagation of action potentials in neurons and other electrically excitable cells (Yu and Catterall, 2003). Generally, neurotoxins are able to modify such action potentials by disrupting the ion channel function. This affects the neuronal excitability and/or neuromuscular transmission in prey organisms and leads to rapid paralysis and subsequent death (Wu and Naharashi, 1988; Messerli and Greenberg, 2006). Neurotoxic polypeptides that target voltage-gated sodium channels are described only for a few marine species. Sea anemones possess 3-to 5-kDa peptides that bind to Na_v channels (Norton, 1991; Homna and Shiomi, 2006). The marine snail *Conus purpurasens* Sowerby produce μ -conotoxin PIIIA that specifically blocks neuronal sodium channels (Safo et al., 2000).

Jellyfish neurotoxins together with other venom compounds are delivered via subcellular stinging organelles, the nematocysts, by which jellyfish capture prey and defend themselves against predators (Heeger et al., 1992; Lotan et al., 1995; Fautin, 2009). Different types of nematocysts were found in tentacles of *C. capillata* medusae. The most common type, the isorhiza, is classified into three different subtypes: 1. A-isorhizas, 2. a-isorhiza, and 3. O-isorhizas (Östman and Hydman, 1997). Isorhizas are broadly described as penetrating nematocysts (Purcell, 1984; Heeger and Møller, 1987; Purcell and Mills, 1988; Östman and Hydman, 1997). To assign a function to different nematocyst types based on their toxic content Wiebring et al. (2010b) separated A- and O-isorhizas as well as euryteles by Laser Microdissection and Pressure Catapulting (LMPC) and analysed them directly with mass spectrometry. Different pattern of potential proteinaceous toxins in a molecular mass range between 4 to 9 kDa were obtained. However, the toxicological characteristics of the presumed toxins were unknown.

The now presented cell assay-guided isolation led to the characterisation of a neurotoxic 8.22 kDa polypeptide. Subsequently, it could be determined in different LMPC–isolated fishing tentacle isorhiza types by matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI ToF MS). The purified neurotoxin specifically blocks receptor site 1 of neuronal Na_v channels. The study contributes to the toxicological characterisation of northern Scyphozoa and enhances the knowledge about the toxic content of certain cnidocyst types and their possible functionality in terms of prey capture.

2. Materials and methods

2.1 Jellyfish material and preparation of nematocysts

C. capillata medusae were collected at the Isle of Lewis [Western Isles (WI), Scotland] and at Rousay [Orkney Islands (OI), Scotland] in 2005. Fishing tentacles of eight medusae (mean umbrella diameter of 36 cm, range 32 – 42 cm) and seven medusae (mean umbrella diameter of 24 cm, range 16 – 31 cm), respectively, were sliced immediately after the animals were caught. The tentacles of each catch were pooled and further prepared for nematocyst extraction in a thermostated laboratory on board of the RV Heincke described by Helmholz et al. (2007). The nematocyst suspensions of WI and OI were immediately stored at –80 °C until further use for venom preparation (WI) and nematocyst separation (WI, OI).

2.2 Venom preparation and peptide isolation

The preparation of the crude venom from thawed nematocyst suspensions of WI, its initial fractionation by size-exclusion chromatography (SEC), the concentration and desalting of SEC fractions, as well as the first purification step by reversed-phase chromatography (RPC1) were carried out according to Lassen et al. (2010). RPC1 fractions of several LC runs (injection volume 100 µL) were combined correspondingly, concentrated and investigated in terms of neurotoxic activity (see 2.3) or stored at –28 °C for further purification. In a second reversed-phase chromatographic step (RPC2), active fractions were purified on the same RP column using the identical elution program described for RPC1 but modified mobile phases (A: 0.1% TFA in ultrapure water, B: 80% acetonitrile with 0.1% TFA). The flow rate, column temperature, and the injection volume were decreased to 0.25 mL min⁻¹, 10 °C, and 40 µL, respectively. Corresponding fractions were again

combined, concentrated in a vacuum centrifuge evaporator (Thermo, Germany), and directly used for the neurotoxicity assay or stored at $-28\text{ }^{\circ}\text{C}$. A final purification step (RPC3) of active fractions was carried out on a Synergi Hydro-RP column (250 x 3.00 mm, 4 μm , 80 \AA ; injection volume 50 μL) utilising the same chromatographic conditions described in the second RP step (RPC2). The resulting isolates were combined, evaporated as described above, and stored at $-28\text{ }^{\circ}\text{C}$ for subsequent mass spectrometric analysis. The detection wavelength in all chromatographic steps was set to 280 nm.

2.3 Tetrazolium-based mouse neuroblastoma cell assay

The mouse neuroblastoma cell line Neuro 2A (CCL 131), purchased from LGC Standards GmbH (Germany) was cultivated in Roswell Park Memorial Institute (RPMI) 1640 cell culture medium (Invitrogen, Germany) with 10% foetal calf serum (FCS; PAA Laboratories, Germany), 100 IU penicillin mL^{-1} , and 100 μg streptomycin mL^{-1} (Sigma, Germany) at $37\text{ }^{\circ}\text{C}$ in a humidified 5% carbon dioxide (CO_2) atmosphere. Cells from a continuous culture were seeded into 96-well microtiter plates at a density of 2×10^5 cells mL^{-1} in 250 μL RPMI 1640 medium per well with penicillin/streptomycin but without FCS and incubated for 24 hours. Culture wells were then inoculated with RPC fractions in dilution series of accordant concentrates. Subsequently, constant volumes (10 μL) of 10 mM ouabain in purified water and 1 mM veratridine in 0.01 M HCl (pH 2) were added. The RPC fractions were added to replicate wells in parallel in the presence and the absence of ouabain/veratridine. Additionally, the following cell controls were performed: untreated negative control (without ouabain/veratridine and without sample), treated positive control (only ouabain/veratridine), and no cell control (culture medium without cells). Each venom fraction and concentration as well as the cell controls were tested in eight replicate wells ($n = 8$) per test.

The prepared plates were incubated for 48 hours. After removing the overlaying medium, 60 μL 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma) solution [2 mM in phosphate-buffered saline (150 mM, pH 7.2; Invitrogen, Germany)] was added to each well. The well plates were incubated at $37\text{ }^{\circ}\text{C}$ for 30 – 40 min. Within the time stated, vital cells were able to reduce the MTT to a blue-coloured derivative. After incubation, 200 μL dimethyl sulfoxide (Sigma) was added to each well for cell lysis and mixed carefully (Manger et al., 1993). The released MTT derivative was immediately measured in a multiwell scanning photometer (Victor 3, Perkin Elmer, Germany) at 550 nm. The cell assay principle and the calculation of the neurotoxic activity (NA) are described in Lassen et al. (2010). In brief, the

NA of each fraction and concentration, respectively, was calculated based on the median absorbance values of the eight replicates per test in the following way: $NA [\%] = (\bar{x}_{Si+v/o} \cdot \bar{x}_{Si}^{-1} - \bar{x}_{Cv/o} \cdot \bar{x}_C^{-1}) \cdot 100$ (\bar{x} : median of the absorbance values ($n = 8$) per test measured at 550 nm; Si+v/o: cell sample treated with venom and ouabain/veratridine; Si: cell sample treated with venom; Cv/o: positive cell control treated with ouabain/veratridine; C: negative cell control).

2.4 Determination of protein contents

The protein content, as a reference value for the venom concentration in the investigated chromatographic fractions, was determined using Bradford's Reagent (Sigma, Germany) in a modified 96-well microtiter plate assay with bovine serum albumin as standard protein and water as dilution agent (Cartelleri et al., 2001). The compound concentrations mentioned throughout the manuscript refer to protein equivalents.

2.5 Nematocyst separation by Laser Microdissection and Pressure Catapulting (LMPC)

Frozen nematocyst suspensions of WI and OI were thawed slowly on ice and macerated again with distilled water on a roller mixer (SRT9; Stuart, United Kingdom) in a cooling chamber (4 °C) for 24 h. The suspension was centrifuged at 1140 x g for 3 min. The supernatant was rejected and the sediment was washed three times with sterile filtered seawater. The resulting nematocyst suspensions were stored at -80 °C or immediately used for the nematocyst separation. The separation of different types of nematocysts was conducted with a PALM[®] MicroBeam (Carl Zeiss MicroImaging GmbH, Germany) into a small drop of distilled water (~ 3 µL), which was placed in the lid of a 0.5-mL Eppendorf tube (Wiebring et al., 2010 a, b). The collected nematocyst samples were stored at -80 °C.

2.6 Mass spectrometry of purified venom fractions and nematocyst samples

Active purified venom fractions were prepared on an AnchorChip[™] target (600/384 T; Bruker, Germany) for matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-ToF MS). The fractions were initially diluted with gradient grade acetonitrile (MeCN; Merck, Germany) at a ratio of 1:1. 0.5 µL of the mixtures were spotted together with 0.5 µL MALDI matrix solution onto the target. The matrix contained 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid; Bruker) in MeCN/0.1% trifluoroacetic

acid (TFA; Fluka, Germany) (90/10 v/v, $c = 1 \text{ mg mL}^{-1}$). The crystallised sample spots were washed with $1 \mu\text{L}$ 10 mM monobasic ammonium phosphate in 0.1% TFA (Merck, Germany). After drying at room temperature the spots were recrystallised with $1 \mu\text{L}$ of a freshly prepared recrystallisation solution [ethanol (gradient grade, Merck)/acetone (picograde, LGC Promochem, Germany)/0.1% TFA (60/30/10 v/v/v)].

The separated nematocyst samples in the lid of the sample tubes were mixed with $3 \mu\text{L}$ of a freshly prepared matrix solution [saturated sinapinic acid in MeCN/0.1% TFA (50/50 v/v)] by centrifugation (30 s, $1140 \times g$). The resulting nematocyst/matrix mixtures ($\sim 4 - 5 \mu\text{L}$) were completely transferred ($4 \times 1 \mu\text{L}$) onto conductive glass slides (Bruker). After crystallisation of the sample spots at room temperature, the glass slides were attached to a MTP Slide Adapter II (Bruker). The target and the slide adapter, respectively, were introduced into the ion source of an Ultraflex II MALDI-ToF mass spectrometer (Bruker). The Ultraflex II was controlled by the FlexControl 3.0 software (Bruker) and externally calibrated with a protein standard (Bruker) containing insulin, ubiquitin, cytochrome C and myoglobin. The samples were analysed in the linear positive mode. Mass signals (m/z) in the spectra were annotated using the FlexAnalysis 3.0 software (Bruker).

3. Results

3.1 Neuroblastoma cell assay-guided isolation of the neurotoxic peptide

The crude tentacle venom, prepared from WI suspensions, was initially fractionated by size-exclusion chromatography and a first reversed-phase chromatographic step (RPC1). A fraction with putative Na_v channel-blocking activity (RPC1-F1) was collected as described by Lassen et al. (2010). Material of RPC1-F1 from eight consecutive runs was combined and concentrated. The resulting concentration was sufficient to perform a dose-effect analysis with the mouse neuroblastoma (MNB) cell assay. A dilution series resulting in six samples of RPC1-F1 exhibited a dose-dependent neurotoxic activity (NA [%]; Fig. 1) in a tested concentration range of $0.5 - 20 \text{ ng protein equivalent well}^{-1}$ (corresponding to $17 - 70 \text{ ng mL}^{-1}$). The detected NA values ranged from 9 to 17% with a maximum activity of 18% at $10 \text{ ng protein equivalent well}^{-1}$ (corresponding to 35 ng mL^{-1}). RPC1-F1 was further fractionated by reversed-phase chromatography (RPC2), yielding eight sub-fractions (Fig.2, RPC2-F1 – RPC2-F8). The measured polypeptide concentrations in all eight RPC2 fractions of 14 combined runs were below the limit of quantification ($< 2 \mu\text{g mL}^{-1}$) of the used adapted

Bradford assay protocol. Decreasing volumes per well of the concentrated fractions, 10, 3.0, 2.0, 1.0, 0.6, and 0.3 μL , [representing dilution series of the concentrated fractions from 1:3.3 (3 μL) to 1:33 (0.3 μL)] were tested with the MNB cell assay. Fraction RPC2-F3 exhibited a clear dose-dependent neurotoxic activity (see Fig. 1). NA values $> 5\%$ were achieved by addition volumes from 1.0 to 10 $\mu\text{L well}^{-1}$. The undiluted fraction (addition volume 10 $\mu\text{L well}^{-1}$) caused a NA of 12%.

The purity of fraction RPC2-F3 was analysed with a final chromatographic step (RPC3). The resulting chromatogram illustrated a distinct component with nearly symmetric peak shape at 9.9 min retention time (Fig. 3). Its approximately 40-fold higher intensity compared to the weak background signals (1 – 6 in Fig. 3) pointed out the isolation of a pure polypeptide which caused the detected neurotoxic effect. Isolates of eight LC runs were combined and concentrated. The purity of the isolated peptide was confirmed by mass spectrometry (see 3.3).

3.2 Separation of nematocysts

Different isorhiza types were isolated by Laser Microdissection and Pressure Catapulting (LMPC) from two different suspensions (WI, OI). An A-isorhiza sample and an O-isorhiza sample, both containing 180 capsules, were obtained from the WI suspension. All three isorhiza types, which were present in the OI capsule suspensions, were separated. The collected samples comprised 64 A-isorhizas, 96 O-isorhizas, and 118 a-isorhizas. The integrity of the sampled isorhizas was checked microscopically after the LMPC treatment.

3.3 Mass spectrometric analysis of the neurotoxin and the separated isorhizas

The isolated neurotoxic peptide was investigated by matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-ToF MS). Due to the fact that sinapinic acid was used as MALDI matrix, relative high laser energies had to be applied to obtain acceptable spectral data. The resulting spectrum substantiated the isolation of a pure peptide which had a quasi-molecular mass $[\text{M}+\text{H}]^+$ of 8217.4 Da (Fig. 4). Because such a neurotoxic polypeptide of *C. capillata* was not yet known it was named CcNT. Two minor signals at a mass-to-charge ratio (m/z) 4110.4 and 6707.1, respectively, were additionally observed. The mass peak at m/z 4110.4 represented the doubly-charged species $[\text{M}+2\text{H}]^{2+}$ of CcNT. The

signal at m/z 6707.1 with approximately 15% of the intensity of the major CcNT signal was likely a degradation product (Fig. 4).

Freshly prepared isorhiza/sinapinic acid mixtures were spotted on glass slides and analysed by MALDI-ToF mass spectrometry. The discharge of the capsules was controlled light-microscopically after preparation on the glass slides. Fig. 5 exemplarily shows a discharged A-isorhiza embedded in sinapinic acid crystals. The resulting mass spectrometric data were sufficient if at least three single spectra from the four prepared spots were achieved. A-isorhizas of the WI suspension exhibited two clusters of peptide signals in the mass range from 4,000 to 6,000 Da and 7,600 to 8,800 Da. Within the narrow mass band, CcNT was clearly detectable (Fig. 6A) but no distinct mass signal of CcNT was observable in the spectra of the accordant O-isorhiza sample (data not shown). Adequate spectra of each isorhiza type were obtained from the collected isorhiza samples of the OI suspension. In all spectra CcNT was traceable. Fig. 6B and Fig. 6C exemplify the results for O- and a-isorhizas, respectively (data for A-isorhiza not shown). In comparison to the spectra of the WI isorhiza samples, slightly expanded clusters of peptides in a mass range from 3,300 – 6,000 Da were visible in the OI isorhiza samples.

4. Discussion

A sodium channel-blocking peptide from the crude nematocyst-derived tentacle venom of the Scyphozoa *Cyanea capillata* is described for the first time. The isolated neurotoxin, named CcNT, with a quasimolecular mass $[M+H]^+$ of 8217.4 Da, specifically blocks voltage-gated sodium (Na_v) channels at receptor site 1. This particular neurotoxic activity of crude *C. capillata* venom on vertebrate neuronal cells was first demonstrated by Lassen et al. (2010). However, this previous study focused on a group of hydrophilic, potentially neurotoxic polypeptides with molecular masses lower than 8 kDa in a partially purified venom fraction. The measured neurotoxic activity (NA) of 11% caused by a concentration of 20 ng well⁻¹ of that former fraction was comparable to the determined NA value of 17% at 20 ng well⁻¹ of fraction RPC1-F1, which was purified in this study using identical chromatographic conditions.

The two-dimensional purification process (Lassen et al., 2010) was extended to a bioactivity-guided three-step process that led to the isolation of CcNT. Due to its hydrophilic property, which can be assumed on the basis of its elution characteristics in RPC2, it is likely that CcNT possesses a number of basic amino acids, e.g. arginine, lysine or histidine. The

presence of strong basic guanidinium groups, e.g. at arginine residues, is required for the ability of neurotoxic compounds to block receptor site 1 of Na_v channels (Shon et al., 1998).

Final reversed-phase chromatography (RPC3) and subsequent mass spectrometry confirmed the purification of the neurotoxic polypeptide. The observable weak signal at *m/z* 6707.1 in the CcNT spectrum was supposed to be a degradation product of CcNT. The decomposition was possibly induced by the high laser energy used for matrix-assisted laser desorption ionisation. Additionally, it is known that jellyfish toxins can be instable and/or thermolabile (Burnett and Carlton, 1987; Anderluh and Maček, 2002). Partial decomposition during the purification of a 31.17 kDa cytotoxin from *C. capillata* was observed by Lassen et al. (2011).

Although the mouse neuroblastoma cell assay has been applied for the detection of neurotoxins from different marine biota, e.g. marine bacteria and dinoflagellates (Gallacher and Birbeck, 1992; Gallacher et al., 1997; Kirchner et al., 2001), this is the first application for a bioassay-guided isolation of a novel jellyfish neurotoxin. Moreover, the assay was sensitive enough to detect a Na_v blocking activity at estimated nanomolar concentrations of CcNT. Neuroblastoma cells of the used cell line Neuro2A express multiple Na_v channel isoforms most of which are Na_v 1.7 channels (Lou et al., 2005). They are known to be blocked by nanomolar amounts of the guanidinium alkaloid tetrodotoxin (TTX) (Goldin, 2002). These facts demonstrate a high receptor specificity of the novel CcNT expressed by very low effective concentrations.

A- and O-isorhizas were separated by Laser Microdissection and Pressure Catapulting (LMPC) from two different nematocyst specimen (WI and OI). Additionally, a sufficient amount of a-isorhizas was isolated from the OI suspensions. High yields of intact capsules were obtained with optimised laser energies according to distinct stability conditions for each isorhiza type described by Wiebring et al. (2010a). A discharge of the capsules occurred during the MALDI preparation, which was obvious after the light-microscopical investigation of the crystallised sample spots on the glass slides. This was probably triggered by the high acid concentrations (trifluoroacetic acid and sinapinic acid) in the capsule/matrix mixtures (pH < 2). Venomous polypeptides, ejected from the capsules or more likely dissolved from the extended tubules, which are able to serve as toxin carrier (Lotan et al., 1996), co-crystallise with sinapinic acid during vaporisation of the solvents. Hence, they were ionisable via the MALDI process and subsequently detectable by mass spectrometry.

The mass spectrometric analysis of the A-, a-, and O-isorhizas showed that CcNT was present in all three investigated types. The capsule types of a- and O-isorhizas prepared from

the tentacles of *C. capillata* are defined as penetrators on the basis of microscopical studies of nematocyst-prey interactions and nematocyst discharge kinetics (Östman and Hydman, 1997, Colin and Costello, 2007) and thus delivering toxins for prey capture. Our confirmation of a venomous peptide with proven neurotoxic activity in both isorhiza types encouraged this assumption. The functional role of A-isorhizas of *C. capillata*, notably described only to entangle prey (Östman and Hydman, 1997; Colin and Costello, 2007) has to be redefined based on our results. Due to the presence of a Na_v channel active neurotoxin in A-isorhizas it is most likely that this nematocyst type also takes part in the envenomation of prey organisms.

5. Conclusion

The newly established neuronal cell assay-guided multidimensional purification process led to the isolation of a novel proteinaceous neurotoxin (CcNT) from *C. capillata* medusae. From the proven Na_v channel blocking activity and its presence in main nematocyst types it was hypothesised that CcNT is one of the fundamental venom compounds involved in the paralysis of prey organisms. Furthermore, the combination of a bioanalytical cell organelle separation with direct molecule-specific mass spectrometric analysis led to the allocation of distinct prey capture functions to *C. capillata* nematocyst types on the basis of their biochemical composition. This study contributes to the knowledge of jellyfish toxins and to the clarification of nematocyst functionality within the Scyphozoa.

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