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Identification of a CAPA-PVK (IXORI-PVK) from single cells of the gulf coast tick, *Amblyomma maculatum*

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Abstract: MALDI-TOF/TOF tandem mass spectrometry has been applied to determine the complete sequence of a CAPA-PVK in the Gulf Coast tick, *Amblyomma maculatum*. Single cell analysis allowed the identification of the amino acid sequence of Ixori-PVK (PALIPFPRV-NH_2), a periviscerokinin which had previously been identified from two other ticks, *Ixodes ricinus* and *Boophilus microplus*. The identification indicates greater conservation of sequence for the CAPA-PVK/CAP2b family in ticks as compared with insects. Side-chain fragmentation experiments provided data to distinguish between Leu/Ile ambiguities. The tick CAPA peptide shows a high sequence homology with other members of the insect periviscerokinin/CAP2b peptides, which are associated with the regulation of critical physiological processes such as diuresis. Thus, the identification of this neuropeptide will provide the experimental basis to better understand regulation of water balance in these arthropods, providing a potential opportunity to develop neuropeptide-based control strategies against these livestock pests.

Keywords: periviscerokinin, CAPA-PVK, MALDI-TOF mass spectrometry, single cell, Ixodidae, diuresis

INTRODUCTION

Members of the family Ixodidae, commonly known as hard ticks, consume large amounts of blood in a single blood meal. As blood feeders, ticks are prominent vectors of diseases caused by a variety of pathogens, including bacteria, viruses, and protozoa; and are responsible for significant economic losses to the livestock industry every year. As obligatory blood-feeding parasites, ticks exhibit highly specialize morphological and physiological adaptations to optimize their food intake strategies. One of these adaptations is the necessity to rapidly remove excess water during a blood meal. Regulation of diuretic processes has also been observed in a number of insects such as mosquitoes [1, 2], blood feeding bugs (e.g. *Rhodnius prolixus*) [3], fleas [4], and lice [5]. It is well established that neuropeptides such as CAPA-periviscerokinins(PVKs)/CAP2bs are key factors in controlling secretion and/or re-absorption processes in the Malpighian tubules and the hindgut [6, 7]. CAPA peptides are neuropeptides typical of the neuosecretory system in the abdominal ventral nerve cord (VNC) which are synthesized in median neurosecretory neurons of the abdominal ganglia and are likely to be released as hormones from the abdominal perisympatic organs (PSOs) into the hemolymph. The designation "capability" (CAPA) originates from the gene´s ability to encode for the neuropeptides related to one of the cardioacceleratory peptides (CAPs) in the VNC/PSOs of the tobacco hawk moth, *Manduca sexta*. Indeed, in a recent study we have identified the first peptidergic neurohormone from ticks, specifically Ixori-PVK (PALIPFPRV-NH_2) from the European hard tick, *Ixodes ricinus* and southern cattle tick *Boophilus microplus* [8].

In this study, the primary sequence of a putative CAPA-PVK from the Gulf Coast tick *Amblyomma maculatum* could be identified by means of direct MALDI-TOF mass spectrometric single cell analysis. *De novo* sequencing yielded an amino acid sequence identical with the CAPA-PVK of *Ixodes ricinus* and *Boophilus microplus* [8].

MATERIAL AND METHODS

Animals. Nymphs of the Gulf Coast tick *Amblyomma maculatum* from a colony originating from ticks collected in Refugio County, TX were fed on poultry at the Tick Research Laboratory (Department of Entomology, Texas A&M University, College Station) and maintained at 23 °C, 85 RH, and a 15 hr photophase. Following ecdysis, adult ticks were transferred to the Areawide Pest Management Research Unit, Southern Plains Agricultural Research Center (USDA-ARS, College Station, TX), where all preparations were performed.

Sample preparation for mass spectrometry. Putative neurosecretory cells were dissected from the synganglion of unfed adult specimens and subsequently prepared for MALDI-TOF mass spectrometric analysis following procedures described in Neupert et al. [8].

MALDI-TOF/TOF tandem mass spectrometry. MALDI analysis was performed on the ABI 4700 proteomics analyzer (Applied Biosystems, Framingham, MA). All acquisitions were taken in manual mode. Initially the instrument was operated in reflectron mode, in order to determine the parent masses. A laser intensity of 3800 was typically employed for ionizing the neuropeptides. For the tandem MS experiments, the CID acceleration used was 1 kV in all cases. The number of laser shots used to obtain a spectrum varied from 500-5000, depending on signal quality. In order to change the net amount of activation energy imparted to the primary ions, the collision gas (atmospheric air) pressure was increased. Three gas pressure levels were employed and labelled by the manufacturer as: 'none', 'medium' and 'high'. The fragmentation patterns from these three different settings were used to determine the sequence of the peptide. The fragmentation data obtained in these experiments was handled using the Data ExplorerT software package.

Immunocytochemistry. Dissected nervous system tissue was fixed for 24 h at 4 °C with 4% formaldehyde in phosphate-buffered saline (PBS), pH 7.2. Subsequently, preparations were washed in PBS-4% Triton X-100 and PBS-1% Triton X-100 for 24 hours, respectively. Then, the preparations were incubated for 3 days at 4 °C in anti-Pea-PVK-2 serum [9] (1:4000) diluted with PBS 1% Triton X-100 containing 0.25% bovine serum albumin and normal goat serum. Following washing in 0.1 mol/l TRIS-HCL, 3% NaCl, 1% Triton X-100 (pH 7.6) for 24 h, the Fluorochrome-labelled (Cy3; Amersham, Braunschweig, Germany) secondary antibodies were directly used as a mixture in PBS-bovine serum albumin (2.5 mg/ml) at a concentration of 1:3000 for 3 days. Finally, the preparations were washed again 24 h in 0.1 mol/l TRIS-HCL, 3% NaCl, 1% Triton X-100 (pH 7.6) and transferred to glycerol. For visualization, tissues were dehydrated in ethanol, cleared in methyl salicylate and mounted in entellan (Euromex microscop Holland).

Documentation. Immunostainings were examined with a confocal laser scanning microscope (ZEISS LSM 510 Meta system, Jena, Germany), equipped with a HeliumNeon1 laser (wavelength 543 nm). Serial optical sections were assembled into combined images. Images were exported and processed with Adobe Photoshop 7.0 software.

RESULTS AND DISCUSSION

The first peptidergic neurohormone from the ticks *I. ricinus* and *B. microplus*, designated as Ixori-PVK (PALIPFPRV-NH_2), had previously been identified by using a combination of immunocytochemistry and mass spectrometric analysis of single cell preparations. In this study, we decided to perform a similar strategy for the identification of putative diuretic hormones in the tick *A. maculatum*. Antiserum raised against the insect diuretic peptide Pea-PVK-II [10] was used to localize neurons in the CNS of *A. maculatum*. Immunocytochemical results yielded a number of PVK-immunoreactive neurons (Figure 1). For single cell analysis, we focussed our interest on a distinct median cell group (T6, cell size: 10-15 μm) located at the posterior end of the CNS. Due to their slightly bluish color (Tyndall effect), we were successful in detecting these neurosecretory neurons in the fresh untreated nervous system. Some of these preparations yielded mass spectra with a distinct ion signal at $[M+H]^+$:1008.64 Da (Figure 2). This ion signal was chosen for fragmentation under conditions of high/medium/low energy CID (Figure 3). The manual analysis of the ion fragment series resulted in the sequence: PALIPFPRV-NH_2, the same amino acid sequence which was identified from *I. ricinus* and *B. mircoplus* [8]. The identification indicates a great conservation of sequence for the CAPA-PVKs in the family Ixodidae. Using information obtained from the CID spectra, even the assignment of Leu/Ile ambiguities could be achieved.

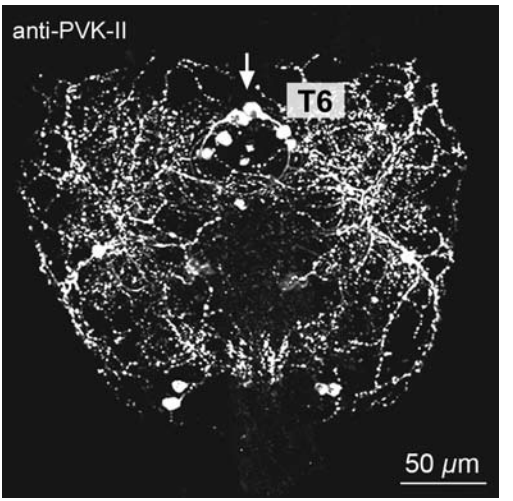

Figure 1. Immunofluorescence staining in whole-mounts of the nervous system from the Gulf Coast tick *Amblyomma maculatum* by means of an anti-Pea-PVK-2 serum. A number of immunoreactive cells could be stained in the synganglion. Single cell preparations were performed on neurosecretory cells which are labelled as T6 neurons (arrow). Scale bar: 50 μm.

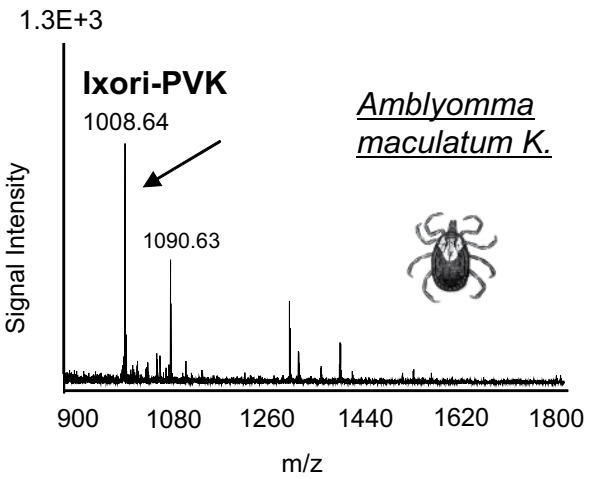

Figure 2. Representative MALDI-TOF mass spectra of a single identified cell from the nervous system of the Gulf coast tick *Amblyomma maculatum* in a mass range of 900-1800 Da. A prominent ion signal at $[M+H]^+$: 1008.63 Da was observed and subsequently fragmented (arrow).

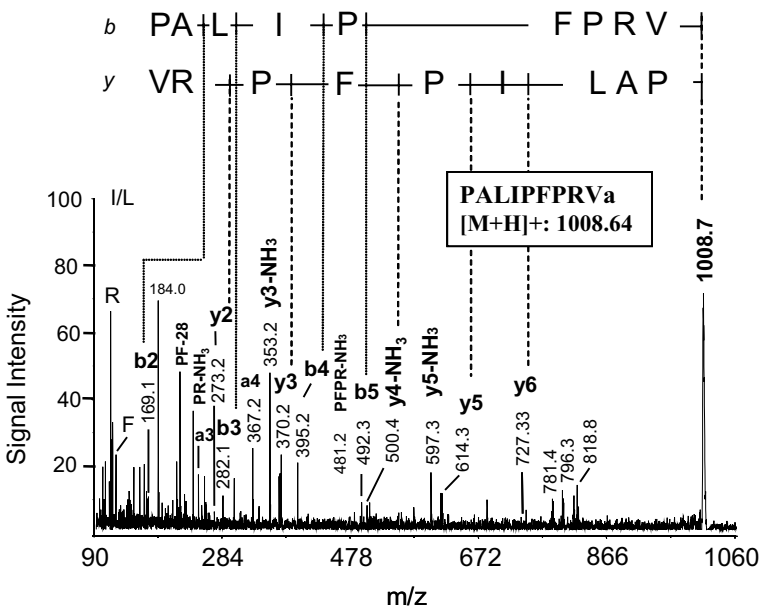

Figure 3. CID mass spectrum of the CAPA-PVK peptide from the Gulf Coast tick, *Amblyomma maculatum*, at $[M+H]^+$: 1008.63 Da under condition of high gas. The fragments were analysed manually and the resulting sequence is given. A number of *y*-, *a*-, and *b*-type fragment ions are labelled which not only enabled the complete *de novo* sequencing but also assignments of internal Leu/Ile (not shown).

The function of this peptide in ticks is still unknown, but the effects of the CAPA-PVK/CAP2b family of peptides in water balance are well established [12]. The tick CAPA-PVK may also regulate diuretic processes in these blood-feeding arthropods. Thus, the sequence information may aid in the future development of mimetic analogs capable of disrupting this critical physiological process in ticks.

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