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Two *capa*-genes are expressed in the neuroendocrine system of *Rhodnius prolixus*

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ABSTRACT

CAPA-peptides have been isolated from a broad range of insect species and are typical of the neurosecretory system of the abdominal ventral nerve cord. In *Rhodnius prolixus,* which is known to transmit Chagas' disease, CAPA-peptides have potent antidiuretic effects. Recently, two *capa*-genes were isolated from this hemipteran insect; it was the first description of the occurrence of multiple *capa*-genes from a single insect species. The expression of peptides from one of these *capa*-genes was confirmed by mass spectrometry Paluzzi (2008) [10]. In this study, the abdominal neuroendocrine system of *R. prolixus* was screened for the occurrence of products of the second annotated *capa*-gene. Single nerve preparations of abdominal segmental nerves 1–3 of *R. prolixus* were analyzed by MALDI-TOF mass spectrometry and the products of both *capa*-genes were identified by tandem mass spectrometry. The co-occurrence of the different CAPA-peptides, including CAPA-precursor peptides, in all spectra from abdominal segmental nerves 1 to 3 suggests a common expression of both *capa*-genes in the same neuroendocrine cells. A comparison of mass spectra obtained from abdominal segmental nerves with those of the subesophageal ganglion (SEG) revealed an obvious differential processing of the CAPA-precursors within the CNS.

1. Introduction

CAPA-peptides have been isolated from a broad range of insect species such as cockroaches, locusts, bugs, beetles, moths, and flies and are typical of the neurosecretory system of the abdominal ventral nerve cord (VNC) (see [17]). These neuropeptides can be grouped into CAPA-periviscerokinins (PVKs) and a CAPA-pyrokinin (PK); both forms were first identified in the American cockroach *Periplaneta americana* [13,14]. The main functions of CAPA-PVKs appear to be the hormonal regulation of diuresis (e.g. [8]) and the modulation of muscle contractions (e.g. [20]). Interestingly, CAPA-PVKs act as diuretic hormones in Diptera [3,12] and as antidiuretic hormones in blood-feeding Heteroptera [9]. To understand and manipulate CAPA-PVK signaling in blood-feeding insect species is potentially of medical importance. The first *capa*-gene was isolated from *Drosophila melanogaster* [7]. For this species, it was shown that the precursor is differentially processed in neurosecretory cells of the abdominal VNC and subesophageal ganglion [15,21]. The *capa*-cells of the abdominal VNC synthesize two PVKs and the CAPA-PK whereas the *capa*-cells of the subesophageal ganglion synthesize only the CAPA-PK, but not the CAPA-PVKs. In the *D. melanogaster* genome, four orthologs (CG8784, CG8795, CG9918, CG14575) of the neuromedin U receptor were identified shortly after its publication [5] The consensus sequence for the vertebrate neuromedin U receptors is LXXPRXa [4], and it was speculated that the different *Drosophila* PRXamides (pyrokinins encoded by *hugin* and *capa*, the two ecdysis-triggering hormones and the CAPA-PVKs) might be agonists of these receptors [5]. Functional expression studies later identified the agonists of the NMU receptor orthologs, and demonstrated that the CAPA-PVKs and the CAPA-PK each have their own specific receptor [2,6,11]. From the large taxon Hemiptera, two PVKs and a single CAPA-PK were identified from different polyphagous stinkbugs by utilizing tandem mass spectrometry [16,18]. Recently, two *capa*-genes were isolated from another hemipteran insect, *Rhodnius prolixus*, which is known to transmit Chagas' disease ([10]; ACH70295). From this species it is known that CAPA-peptides have potent antidiuretic effects [9,10] which may prevent excessive loss of water after the postgorging diuresis that is typical of hematophagous insects. The expression of peptides from one of these *capa*-genes (*capa*-a-gene) has been confirmed by mass spectrometry [10].

In this study, the abdominal neuroendocrine system of *R. prolixus* was screened for the occurrence of products of the second annotated *capa*-gene. Specifically, abdominal neurohemal release sites, which in insects are commonly referred to as perisympathetic organs (PSOs), were used. In Hemiptera, these organs belong

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to the evolved type that is not linked with median nerves [1]; immunostaining verified their nature as neurohemal release sites [9,16]. The primitive type of abdominal PSOs that is linked with well developed median/transverse nerves persists in a few Hemiptera (e.g. spittlebugs; Pergner and Predel, unpublished), but most of these insects do not develop median nerves and the release sites migrate to the base of abdominal segmental nerves 1–3 that probably incorporate the transverse nerves. The peptidome of these nerves in *R. prolixus* was analyzed and the products of both *capa*-genes identified.

2. Materials and methods

2.1. Animals

Adult *Rhodnius prolixus* Stâl were provided by Professor Ian Orchard (University of Toronto, Canada) and Dr. Wolfgang Nentwig (Bayer AG, Leverkusen, Germany). Only unfed adult insects of both sexes were used throughout the experiments.

2.2. Dissection and sample preparation for mass spectrometry

Insects were fixed upside down with insect pins and the sternites removed with scissors. The fused ventral nerve cord and the brain with the retrocerebral complex were isolated and placed in a separate chamber filled with insect saline (pH 7.25) of the following composition: NaCl (7.50 g/l), KCl (0.20 g/l), $CaCl_2$ (0.20 g/l) and $NaHCO_3$ (0.10 g/l). Using fine scissors, the abdominal nerves 1 to 3 and the reterocerebral complex were separated from the ganglionic mass and subsequently transferred (with an insect pin) into a drop of water on a stainless steel sample plate for MALDI-TOF mass spectrometry. After few seconds, the water was removed using a glass capillary. The subesophageal ganglion (SEG) tissue was cut in small pieces and transferred with a glass capillary into a drop of distilled water on the same sample plate and subsequently treated as described above. Approximately 100 nl of matrix solution (saturated α-cyano-4-hydroxycinnamic acid dissolved in methanol/water [2:1]) was injected onto the dried tissues over a period of about 5 s using a Nanoliter injector (World Precision Instruments, Berlin, Germany). Each preparation was air-dried again and covered with pure water for a few seconds, which was subsequently removed by cellulose paper. A detailed description of the direct profiling of brain tissue is given in [19].

2.3. MALDI-TOF mass spectrometry

MALDI analysis was performed on the ABI 4800 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. For the tandem MS experiments, the CID acceleration was 1 kV in all cases. The number of laser shots used to obtain a spectrum varied from 600 to 4000, depending on signal intensity. Three gas pressures were employed: none, medium and high. The fragmentation patterns from these three different settings were used to determine the sequences of the peptides. The fragmentation data obtained in these experiments was processed using the Data ExplorerT software package.

3. Results

Single nerve preparations of abdominal segmental nerves 1 to 3 of *R. prolixus* were analyzed by MALDI-TOF mass spectrometry. The mass spectra revealed prominent ion signals (see Fig. 1) which were mass-identical with the theoretical monoisotopic masses ($[M+H]^+$) of the three known products of the first *capa*-gene

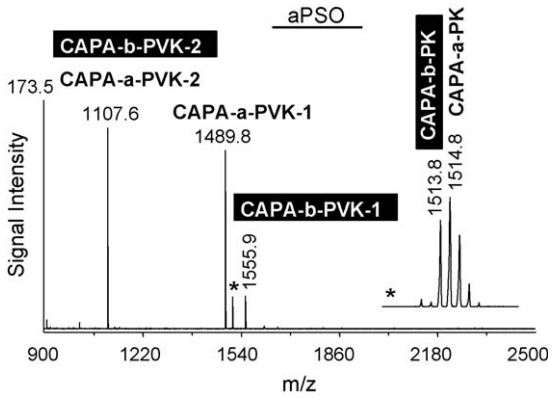

Fig. 1. MALDI-TOF mass spectrum typical of an abdominal segmental nerve preparation of *R. prolixus* (abdominal segmental nerve 2 is shown). Note that the CAPA-peptides of both *capa*-genes are detectable. Products of the CAPA-a precursor (dark labeling on white background) and CAPA-b precursor (white labeling on dark background) are labeled.

(ABS17680). In addition, two further ion signals (1513.8, 1555.9), which were mass-identical with predictable products of the second *capa*-gene (ACH70295), were observed. The sequence of CAPA-PVK-2 is identical in both *capa*-genes. The putative neuropeptides of the second *capa*-gene were subsequently fragmented (see Fig. 2 for CAPA-b-PVK-2). The mass difference of the CAPA-PKs from the different *capa*-genes is only one Da and, in MALDI-TOF/TOF mass spectrometry, an ion gate of more than one mass unit is usually required for fragmentation. For that reason the two PKs were not fragmented separately (Fig. 3). Nevertheless, the fragment analyses unambiguously confirmed the expression of both PKs in neurons that provide the abdominal segmental nerves 1 to 3 with neurosecretions. The mass fingerprints (Fig. 1) suggested that the products of both genes are accumulated roughly in the same range in the neurohemal tissue. By mass-matching, further putative products of the CAPA-precursors (Fig. 4) were determined, namely the precursor peptides (CAPA-PPs) that are located in the precursor sequence between the two PVKs as well as an extended form of CAPA-PK (Fig. 5A). Low signal intensity of these peptides precluded fragment analysis. The complete sequences of the CAPA-peptides of *R. prolixus*, which were detected by MALDI-TOF mass spectrometry, are summarized in Table 1.

Mass spectra of pieces of the SEG revealed the occurrence of CAPA-peptides in a number of samples. However, only the CAPA-PKs and putative CAPA-PPs were detectable in these spectra (Fig. 5B). Weak signals typical of the CAPA-PKs were also observed in spectra from the retrocerebral complex (not shown).

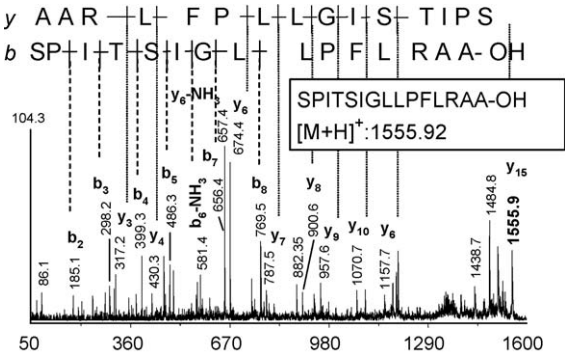

Fig. 2. CID mass spectrum of the peptide at $[M+H]^+$: 1555.9 Da. The fragments were analyzed manually and the resulting sequence is given. A number of *y*-, *b*-type ions are labeled which confirm the amino acid sequence of CAPA-b-PVK-1.