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Serine phosphorylation of CAPA pyrokinin in cockroaches—A taxon-specific posttranslational modification

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ABSTRACT

In insects, posttranslational modifications of neuropeptides are largely restricted to C- and N-terminal amino acids. The most common modifications, N-terminal pyroglutamate formation and C-terminal α-amidation, may prevent a fast degradation of these messenger molecules. This is particularly important for peptide hormones. Other common posttranslational modifications of proteins such as glycosylation and phosphorylation seem to be very rare in insect neuropeptides. To check this assumption, we used a computer algorithm to search an extensive data set of MALDI-TOF mass spectra from cockroach tissues for ion signal patterns indicating peptide phosphorylation. The results verify that phosphorylation is indeed very rare. However, a candidate was found and experimentally verified as phosphorylated CAPA pyrokinin (GGGGpSGETSGMWFGPRL-NH_2) in the cockroach *Lamproblatta albipalpus* (Blattidae, Lamproblattinae). Tandem mass spectrometry revealed the phosphorylation site as Ser^5. Phosphorylated CAPA pyrokinin was then also detected in most other cockroach lineages (e.g. Blaberidae, Polyphagidae) but not in closely related blattid species such as *Periplaneta americana*. This is remarkable since the sequence of CAPA pyrokinin is identical in *Lamproblatta* and *Periplaneta*. A consensus sequence of CAPA pyrokinins of cockroaches revealed a conserved motif that suggests phosphorylation by a Four-jointed/FAM20C related kinase.

Introduction

Posttranslational modifications (PTMs) of proteins are necessary for the regulation of almost all cellular processes (e.g. protein activity, turnover rate and intracellular trafficking). Their structural diversity results in a high diversification of the proteome [32,60]. Among PTMs, reversible phosphorylation of proteins is a particularly ubiquitous mechanism, important for many signaling pathways and catalytic activities [8,33,36]. The functional importance of such phosphorylations is emphasized by the proportion of protein kinases encoded within eukaryotic genomes [34].

In contrast to PTMs of proteins (including protein hormones), PTMs of neuropeptides are largely restricted to the cleavage of precursor proteins [23] and protective PTMs of the terminal amino acids. The most common of these protective modifications are N-terminal pyroglutamate formation and C-terminal α-amidation [5,7,50]. Other PTMs which are occasionally found in neuropeptides include e.g. hydroxylation, sulfation, acetylation, glycosylation, and phosphorylation [3]. In insects, sulfation is typical of sulfakinins [38], which share sequence homology with vertebrate gastrins/cholecystokinins. Glycosylation, hydroxyprolination, acetylation and phosphorylation of insect neuropeptides, however, have been identified in a few cases only; specifically in adipokinetic hormone from the stick insect *Carausius morosus* (glycosylation; [37]), the stink bug *Nezara viridula* (hydroxyprolination; [19]), the beetle *Trichostetha fascicularis* (phosphorylation; [18]); also in head peptide from the mosquito *Aedes aegypti* (hydroxyprolination; [59]), callatostatin-5 of the blowfly *Calliphora vomitoria* (hydroxyprolination; [16]) and in a FMRF-amide related peptide from the kissing bug *Rhodnius prolixus* (acetylation; [39]). It is not yet clear if these findings reflect a nearly complete absence of such PTMs in insect neuropeptides or point to poor knowledge regarding that topic. Genome sequences do not provide insight into that question.

In this study, we used a computer algorithm to search an extensive data set of mass spectra from neurosecretory organs of cockroaches for characteristic mass shifts and neutral losses indicating phosphorylations. This screening confirmed that phosphorylation of insect neuropeptides is indeed rare. With CAPA

Abbreviations: MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; PK, pyrokinin; PTM, posttranslational modification.

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pyrokinin (CAPA-PK) of *Lamproblatta albipalpus*, however, a candidate neuropeptide with phosphorylation was identified. By using preparations of abdominal perisympathetic organs (aPSOs), the phosphorylation as well as the site of phosphorylation could be verified by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. Surprisingly, this PTM was found to be taxon-specific. In closely related cockroaches such as *Periplaneta americana* and *Blatta orientalis* we did not observe this modification although the CAPA-PK sequences are identical in all of these insects. On the other hand, phosphorylated CAPA-PK was identified in cockroach taxa which are not closely related such as blaberid and polyphagid cockroaches.

Materials and methods

Insects

Cockroaches were reared under a 12 h light, 12 h dark photoperiod at a temperature of 26–28 °C. Animals had free access to water and were fed with complete feed for mice, vegetables and fruits. Only adult specimens of both sexes were used for experiments.

Dissection and sample preparation for MALDI-TOF mass spectrometry

For dissection of aPSOs, cockroaches were fixed with insect pins. Body caveats were opened from ventral and sternites were removed with forceps and scissors. The abdominal ventral nerve cord with attached PSOs was isolated by removing adherent tissues and subsequently transferred into a separate dish filled with insect saline (pH 7.4, containing 7.50 g/l NaCl, 0.20 g/l KCl, 0.20 g/l $CaCl_2$, 0.10 g/l $NaHCO_3$). Abdominal PSOs were then cut off and either transferred to a MALDI sample plate for direct tissue profiling or into 5 μl of 0.1% trifluoroacetic acid (TFA). Extraction of PSO peptides in 0.1% TFA was performed by sonication (several times for 10 s) in a chilled water bath and subsequent centrifugation.

For direct tissue profiling, 0.3 μl of matrix solution was added to dried samples and allowed to air dry. In case of PSO extracts, 0.3 μl of sample solution was mixed with 0.3 μl of matrix solution on the sample plate and allowed to dry. All preparations were finally washed with a drop of pure water for a few seconds to reduce salt contamination.

Matrix stock solution was prepared by dissolving α-cyano-4-hydroxycinnamic acid (CHCA, Sigma–Aldrich, Steinheim, Germany) in ethanol/acetonitrile/water (60:36:4, v/v) to a final concentration of 10 mg/ml. Working solutions were freshly prepared by dilution with water/methanol (50:50, v/v) at a ratio of 1:1 (v/v).

Synthetic peptides and phosphatase treatment

Phosphorylated CAPA-PK was purchased from Peptide 2.0 Inc. (Chantilly, USA). Pea-sulfakinin (EQFDDY(SO_3)GHMRF-NH_2) was synthesized as described in [42]. Phosphatase incubation mixture was prepared by dissolving alkaline phosphatase solution from bovine intestinal mucosa (Sigma–Aldrich) in 50 mM ammonium bicarbonate buffer (pH 8.2) to a final concentration of 0.1 U/μl. For experiments with synthetic peptide, 1 μl phosphorylated CAPA-PK (10^{-4} M) in 0.1% TFA was incubated with 15 μl phosphatase incubation mixture at 37 °C for 30 min. For experiments with native CAPA-PK of *L. albipalpus*, an extract of 5 aPSOs in 5 μl of 0.1% TFA was incubated with 5 μl phosphatase incubation mixture at 37 °C for 30 min. Following incubation, samples were acidified by addition of 1.5 μl (synthetic peptide) or 0.5 μl (native peptides) 10% TFA to stop enzymatic activity.

MALDI-TOF mass spectrometry

MALDI-TOF mass spectra were acquired using an ABI 4800 proteomics analyzer (AB SCIEX Germany GmbH, Darmstadt, Germany) or an UltrafleXtreme TOF/TOF mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany). All MS acquisitions were made under manual control in reflector positive ion mode in a detection range of *m*/*z* 600–3000. Instruments were calibrated using Bruker peptide standard kit. Data obtained in these experiments were processed with the Data Explorer software Version 4.3 (Applied Biosystems) or FlexAnalysis 3.4 software package (Bruker). MS/MS experiments were performed by laser-induced fragmentation without application of a collision gas. Peptide identities were verified using MS/MS fragmentation of the molecules, determination of the molecular mass of the fragments, and comparison of predicted (http://prospector.ucsf.edu) and experimentally obtained fragmentation patterns.

Search algorithm for the detection of phosphopeptides

An existing data set of mass spectra from neurohemal tissues (mainly corpora cardiaca, thoracic and abdominal PSOs) of cockroaches (more than 60 species belonging to all major clades, including termites) was screened by an algorithm written in Visual Basic for Applications (Microsoft Visual Basic 6.0) for a specific pattern of ion signals with mass shifts typical of phosphorylation (+80 Da) and the respective sodium (+22 Da) and potassium (+38 Da) adduct ions as well as neutral losses of phosphoric acid (−98 Da). For this purpose, monoisotopic peak tables were exported from Data Explorer and pasted into separate excel sheets (Microsoft Excel 2002) for subsequent analysis. The scanning algorithm searched for peaks with a mass difference of 80 Da. To reduce false positive results, the probability of a mass difference resulting from phosphorylation was estimated by the following criteria. The first criterion was the presence of a less prominent ion signal with reduced resolution and a mass difference of −98 Da; indicating neutral loss of phosphoric acid by post source decay. The second criterion was that the putative phosphopeptide has a pattern of corresponding sodium and potassium adduct ions, and that these ions have lower signal intensity than the protonated ion species. All calculations based on mass differences were performed with a mass tolerance of 0.1 Da except for the detection of the neutral loss of phosphoric acid, where 1.5 Da was applied.

Results

Screening a library of mass spectra from preparations of neurohemal organs of cockroaches

A library of MALDI-TOF mass spectra (CHCA spectra, *m*/*z* 800–3000) from neurohemal organs of cockroaches was screened with a search algorithm that was adapted to detect ion signal patterns typical of phosphopeptides. This approach yielded a distinct hit in mass spectra of aPSOs from *L. albipalpus* (Blattidae, Lamproblattinae). The ion signal at *m*/*z* 1731.8 was recognized as putative phosphorylated product of a peptide mass-identical with *P. americana* CAPA-PK at *m*/*z* 1651.8. The sequence of this CAPA-PK is typical of a number of blattid cockroaches [53]. Spectra of aPSO-preparations from *L. albipalpus* were reproduced by direct tissue profiling (Fig. 1).

Confirmation of phosphorylation

Beside phosphorylation (+80 Da; more exact +79.966 Da), sulfation is another PTM resulting in a similar mass shift (+79.957 Da). In MALDI-TOF mass spectrometry, however, sulfation is not