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Expression and possible functions of PACAP-like and CAPA peptides in mature specimens and embryos of *Eisenia fetida*

PhD-thesis

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Introduction

Classification, distribution and observation of neuropeptides in invertebrates

Neuropeptides such as neurotransmitters, neuromodulators and neurohormones as signal molecules have pleiotropic function in various animals. The members of neuropeptide-families are characterized by different amino acid sequence conservation and phylogenetic pattern. Certain neuropeptides, proved to be conservative in sequence and function (e.g. oxytocin/vasopressin-like, and FMRFamid-like peptides) thus they were identified in various species of both invertebrates and vertebrates. Identified receptors of invertebrate neuropeptides belong to the G-protein-coupled receptors (GPCR) and they are similar to GPCRs of vertebrates. Introduction of immunohistochemistry to neurobiology facilitated the identification of neuropeptides and their function. Expression of several neuropeptides, known to be vertebrate neuropeptides earlier, was investigated in some invertebrate model-animals and some of them (e.g. pituitary adenylate cyclase activating polypeptide, PACAP) occurred in the central nervous system (CNS) of invertebrates and the earthworm *Eisenia fetida* as well. Later on the amino acid sequences of certain peptides expressed in invertebrates (e.g. FMRFamide-like and oxytocin-like peptides) were determined, too. Contrary to the technical development of the isolation, purification and sequence analysis only few neuropeptides had been identified in Annelids.

In our research project we investigated the occurrence, distribution and possible effects of certain conservative neuropeptides (PACAP and CAPA peptides) in the earthworm *E. fetida* that is a model animal of comparative neurobiology and developmental biology because of the simple organisation of its CNS and the easy dissection of its embryos.

PACAP and its specific receptor (PAC1R) in vertebrates and invertebrates

Two isoforms of PACAP (PACAP 38 and 27) were isolated from the ovine hypothalamus. It has a wide influence on various organs of matured specimens of vertebrates, in addition it is a pleiotrop regulatory factor during the embryonic development. The expression of PACAP-like and PAC1R(-like peptides) in some invertebrates (e.g. *Drosophila. melanogaster, Helix pomatia, Lymnaea stagnalis,*) furthermore in an oligochaete worms (*Lumbricus polyphemus*) had been shown.

Their occurrence in the central and peripheral nervous system and peripheral organs and tissues (hemolymph, the wall of hemolymph lacunes and salivary glands of molluscs) had been proved.

The expression of PACAP, which is known to be a pleiotropic neuropeptide, has been investigated by immunohistochemistry and bioassay in the earthworms but its possible effects in adult worms and in earthworm embryos remained unknown.

Expression and functions of CAPA peptides in invertebrates

Our research was expanded to the investigation of CAPA peptides, thought to be insect neuropeptides earlier, which are grouped to periviscerokinins (PVKs) and pyrokinins (PKs) based on their sequence-homology. Both groups are characterized by a conservative C-terminal amino acid motif such as PRVamide for PVKs and FXPRLamide for PKs.

These peptides are mainly synthesized by neurosecretory cells in insects and they act as neurohormones, however, they are occurred in certain interneurons as well. To our best knowledge no CAPA peptide or similar sequences were determined in annelids that is phylogenetically a sister group of insects.

Aims

To establish the possible function of PACAP and its specific receptor (PAC1R), we feel it necessary to

1. determine of the three dimensional pattern of PACAP and PAC1 relative peptides, which are expressed in annelids, in the CNS of mature and juvenile specimens of the *E. fetida*;
2. investigate the ultrastructural localization and molecular characteristics of the PAC1R-like peptide;
3. observe the occurrence of PACAP-like peptides and determination of its concentration kinetic in embryos, and in those tissues and organs (e.g. clitellum) that play a role in of earthworm reproduction;

Focusing on CAPA peptides that are thought to be characteristic for insects we would like to know

1. whether CAPA peptides or similar molecules are expressed or not in annelids that are a phylogenetically sister group of insects;
2. which amino acid sequences are characteristic for the putative CAPA peptides expressed in earthworms;
3. what the pattern of the CAPA peptide expressing structures in the CNS and peripheral tissues of developing and mature earthworms is; and
4. what the cellular localization of the PVK/PK-like peptides is in the CNS of mature worms?

Materials and methods

All of the experiments were carried out on embryos and mature specimen of the manure worm (*Eisenia fetida* Sav. Annelida, Oligochaeta). Embryos were classified into five distinct groups (E0-E4) based on their morphological characteristics (amount of vitelline, the number of closed segments, the presence of the main blood vessels). In some experiments isolated tissues or organs of BALB/c mouses (*Mus musculus*) were applied as controls.

Immunhistochemistry and immuno electronmicroscopy

Pieces of dissected ventral nerve cord of mature worms and isolated embryos were fixed in buffered fixatives (4% paraformaldehyde, 0.25% glutaraldehyde containing 4% paraformaldehyde, or Boer-fixative) according to the investigated antigens.

Pre-embedding immunhistochemistry

After fixation, washing and permeabilization of tissues they were incubated in one of the primary antibodies (anti-PACAP27, anti-PAC1R, anti-Pea-PVK II). Labelled structures were visualized by two different methods. Some samples were labelled with fluorescent secondary antibody and observed with a fluorescent microscope or a confocal laser scanning microscope. Other samples were labelled with avidin-biotinylated peroxidase complex (ABC), ExtrAvidin kit and visualized by 3,3'-diaminobenzidine (DAB). Serial sections were made by a cryostat from a part of PAC1R and PVK/PK-labelled samples.

Post-embedding immunohystochemistry

For light microscopy and electron microscopy the samples were dehydrated and embedded into paraffin or epoxy-wax. According to the histo- and cytochemical protocols the sections were treated with reagents and the localization of antigens (PAC1R) was visualized by DAB or immunogold (18 nm), contrasted as usual and observed by a light or electron microscope.

Biochemical observations

Determination of PACAP concentration with radioimmunoassay (RIA)

After homogenization and centrifugation of tissue samples the supernatants were used for RIA. To each polypropylene tube containing 0.05M- phosphate buffer added 100 µl diluted antiserum (anti-PACAP), 100 µl RIA tracer (radioactive iodine labelled PACAP fragment) and 100-100µl of known concentrations of synthetic PACAP38 or the investigated samples.

Following the incubation the antibody-peptide complex was separated from the free antibodies and its radioactivity was determined by a gamma-counter.

Semiquantitative peptide analyses of cocoon-fluid by Dot blot

After homogenization and centrifugation of collected cocoon-fluid the supernatants were placed and dried to polyvinyl-difluoride membranes and incubated with a blocking solution, then treated with anti-PACAP solution, washed in a buffer and labelled with infrared stain conjugated IgG. The infrared intensity of samples was detected with an infrared counter.

Western blot, immunoprecipitating western blot and far western blot

All of the experiments were carried out on supernatants of homogenized and centrifuged samples. For immunoprecipitation of PAC1R-like peptides the supernatants were purified by affinity chromatography applying anti-PAC1R antibody coupled to protein-G-Sepharose beads. For conventional WB and far WB supernatants were twofold diluted with Laemmli-buffer and heated.

Following gel electrophoresis (10 % polyacrylamide gel containing sodium-dodecyl-sulphate) the separated peptides were transported on a nitrocellulose membrane. For far-WB the membranes were incubated in synthetic PACAP38 peptide containing solution, washed and treated with anti-PACAP solution. For WB and IP-WB the membranes were incubated in anti-PAC1R solution. In all the experiments horse-radish peroxidase conjugated IgG was used as secondary antibody and labelled bands were visualized with ECL (enhanced chemiluminescent) reagent and detected by a light-sensitive film.

Purification of CAPA peptides and their identification with mass spectrometry

Supernatant of *E. fetida* tissue homogenates were purified with affinity chromatography applying IDM protein-A beads conjugated with Pea-PVK II antiserum. The bounded peptides were eluted with 50% (v/v%) acetonitril -0.5% trifluoroacetic-acid and investigated by mass spectrometry. The eluted PVK/PK peptides were cleaned with online reverse phase nanoscale capillary liquid chromatography and the fractions were analysed with electrospray tandem mass spectrometry. From the mass data the amino acid sequences of peptides were determined by manual *de novo* sequence analysis.

Results and discussion

The functional anatomy of PACAP- and PAC1R-like peptides in mature and developing earthworms

PACAP- and PAC1R-IR neurons were identified as small interneurons of the ventral nerve cord in mature earthworm based on the anatomical position of perikarya and pathways of their processes. We described two interganglionic fibre-bundles which were identified as interneuronal and sensory ones.

Varicose PACAP-IR fibres run close to the PAC1R-IR fibres and PACAP containing vesicles were found in several synapses, suggesting that PACAP or PACAP-like peptide acts as neuromodulator or a neurotransmitter in the interneuronal system of earthworms.

High number of PAC1R-IRsensory fibres occurred in close vicinity the 3rd segmental nerves and enter the ventrolateral, ventromedial or the intermediomedial sensory longitudinal axon bundles of the ipsilateral hemiganglia. The 3rd segmental nerves and two sensory longitudinal axon bundles (ventrolateral and intermedio-medial) contained PACAP-IR neural processes as well, which means PACAP(-like peptide) acts as neuromodulator in both the peripheral and central parts of the sensory system.

Those PAC1R-IR neurons located near the root of the 3rd segmental nerves were identified as small motoneurons and central sensory neurons, while others could function as neurosecretory cells.

By means of immunohistochemistry the first detectable PACAP-IR neurons were only found in the CNS of the last developmental stage embryos (E4) close to the hatching. In contrast to this the first PAC1-IR structures occurred in E1 developmental stage both in the developing CNS and neural structures of the body wall. Each ventral nerve cord ganglion had a few PAC1R labelled cells and the antero-posterior directed polysegmental fibre bundles. No segmental nerves of any developing embryos contained PACAP or PAC1R-IR fibres, suggesting that during the embryonic development these peptides are only expressed in interneurons. The huge difference between the pattern of PACAP and PAC1R immunoreactivity of hatched and mature specimens strongly suggest a remarkable postembryonic development of PACAP and its specific receptor in earthworms.

Several spherical PACAP-IR and PAC1R-IR cells without visible processes were seen in the body wall of E1 developmental stage embryos by a light microscope. We could propose from this finding that PACAP(-like peptide) has a paracrine effect at the periphery during embryonic development.

Later on (E3 and E4 developmental stages) columnar primer sensory cells with well visible processes were found at the same anatomical positions where PAC1R-IR labelled spherical cells occurred which means PACAP(-like peptide) mediates the differentiation of primary sensory cells.

A subepidermal plexus formed by PACAP-IR fibres was found in the developing mouth, and we can conclude that PACAP(-like peptide) plays a role in the chemosensory processes in earthworms, similarly to other invertebrates.

Ultrastructural localization of PAC1 receptor in developing and mature earthworm

Ultrastructural investigations showed that PAC1R localized to granular endoplasmic reticulum (GER) cisternae, vesicles characterized by various size and density and plasma membranes of neurons of both developing and mature earthworms. Based on these findings we could propose that the PAC1R(-like peptide) synthesized in GER cisternae and were transported to the plasma membrane by vesicular transport.

Several synapses stained for PAC1R. Both pre- and postsynaptic membranes of the symmetrical synapses strongly labelled, indicating that PACAP(-like peptide) could act as neurotransmitter and neuromodulator in the CNS of *E. fetida*. Plasma membrane bounded PAC1R immunoreactivity was only found on the processes of small dark neurons identified as sensory neurons.

In mature worms the neural submembrane cisternae were also stained, indicating a possible extrasynaptic depletion of PACAP.

Since the endothel of the small blood vessels was PAC1-IR too, there is a possibility that PACAP mediate the blood distribution in earthworms.

Quantitative and semiquantitative measurement of PACAP and PAC1R expression in *E. fetid*a

Applying radioimmunoassay (RIA) we showed that the concentration of expressing PACAP(-like peptide) was almost the same during the embryonic development from E1 stage to E3 one, but its concentration elevated three times in E4 stage embryos.

By means of dot blot occurrence of PACAP-IR compounds were shown in the cocoon-fluid surrounding the zygote (E0 developmental stage), in which PACAP concentration decreased continuously untill E4 developmental stage.

RIA experiments proved that PACAP(-like peptide) was also present in the body wall of mature worms and its concentration was the highest in the clitellum that secrete cocoon fluid (albumen), suggesting that PACAP(-like peptide) played a role in reproduction and

embryogenesis of *E.fetida*.

The PACAP(-like peptide) concentration of the blood was tenfold that was found in the ventral nerve cord. This compound was probably synthesized by those neurons located close to the root of the 3rd segmental nerves identified neurosecretory cells. High PACAP(-like peptide) concentration in the blood and occurrence of PAC1R(-like) immunoreactivity in the blood vessel endothel can imply neurohormone role and vasoactive function of PACAP(-like peptide) in earthworms.

The molecular weight of PAC1R(-like protein), determined by WB and IP-WB, was about 50 kDa. Applying Far WB we found that there is a synthetic PACAP38 binding molecule in earthworm also having 50 kDa molecular weight.

Based on our results we concluded that a PAC1R immunoreactive molecule was expressed during the whole life cycle of *E. fetida* and it could be a newly identified splice variant of the PAC1R in earthworms. It is well known that the size, molecular weight of PAC1R is various in vertebrates and the identified splice variants significantly differed from each others. We propose from the results that (PACAP(-like peptide) of *E. fetida* is pleiotropic in function and influence the development further it acts as neurotransmitter and neuromodulator as well.

Identification and sequence analysis of PVK/PK relative peptides in *E. fetida*

By means of mass spectrometry using of the immunoprecipitated compounds of the *E. fetida* ventral nerve cord six new neuropeptides (SPFPRI/La, APFPRI/La, SPLPRI/La, SFVRI/La, AFVRI/La, SPAFVRI/La) were identified. Based on the conservative C-terminal sequences they were named XRI/Lamides. These peptides were similar in composition to the identified insect and mollusc periviscerokinins and pyrokinins, implying that these are ancient molecules developed during the evolution.

Characterization of XRI/Lamide expressing neural system in the ventral nerve cord of mature *E. fetida*

The number of IR perikarya ranged from 112 to 124 in each ganglion and no significant differences were found in various portion of the ventral nerve cord. Neurons with heterogeneous morphology and neural process pathways localized to the posterior part of ganglia. Most of the labelled fibres situated in interganglionar longitudinal fibre bundles. Besides of the numerous ipsilateral processes a few crossing posteriorly directed ones were also identified. All segmental nerves contained labelled nerve processes.

Based on the anatomical position of labelled perikarya and their processes IR neurons could belong to small interneurons and motoneurons. Ultrastructural investigations revealed that

6 different neuron types of labelled neurons could be distinguished based on their cytological characteristics. Those cells situated between the roots of 2nd and 3rd segmental nerves showed all cytological characteristics of neurosecretory cells.

Pattern of XRI/Lamide IR structures in developing embryos of *E. fetida*

Antero-posteriorly directed development and the increase in the number of labelled neurons and a gradual differentiation of the interganglional fibre bundles of the central nervous system were characteristic during the embryonic development. Most of the occurred neurons were polysegmental or local interneurons, however neurosecretory cells close to the roots of 3rd segmental nerves were also identified. The pattern of these cells did not change during the postembryonal development.

In contrast the XRI/Lamide(-like) peptides expressing cells of the body wall consist of a permanent and a transient populations. The latter group did not occur in the hatching worm yet.

The first cells of permanent population occurred in the anteriormost segments of embryos at E2 developmental stage. The most characteristic structures of each segment were a pair of bipolar cells with centrally directed processes which enter the ipsilateral hemiganglion so they were identified as primary sensory cells.

Labelled cells of the transient populations were uni- or bipolar ones that occurred in the posteriormost segments and were visible in E1 developmantal stage embryos. A part of the neural processes enter the developing central nervous system.

We detected first a square network consisting of labelled neural processes in the body wall of developing embryos. Some of the processes encircled the chaete-sacks others enter the segmental nerves. This system probably mediates neural signals of the mechanoreceptors and/or motor command of the segmental ganglion to the retractor and protractor muscles of chaete-sacks. This network could not be seen in the body wall of mature earthworms, probably the thickening body wall inhibits the diffusion of immunocytochemical reagents into whole mount preparation.

Any similar network could not be found yet either in polychaetes, oligochaetes, or hirudinoids. To establish the exact organization of its structure and possible functions further investigations are needed.

Summary

Neuropeptides such as neurotransmitters, neuromodulators and neurohormones as signal molecules have pleiotropic function in both developing and mature animals. This work focused on the functional anatomy of conservative neuropeptides (pituitary adenylate cyclase activating polypeptide, PACAP; and CAPA-peptides like periviscerokinines and pirokinines) expressing neural structures in both developing and mature specimens of *E. fetida*.

We established the expression of both PACAP(-like peptides) and PAC1R(-like peptide) during the whole life cycle of *E. fetida*. Presence and changes in concentration of these peptides were investigated by radioimmunoassay, dot blot, immunohistochemistry further conventional, immunoprecipitating and far Western blots. PACAP(-like peptide) was detectable not only in developing embryos but also in cocoon-fluid (albumen). The albumen secreting structure the clitellum and the blood of matured earthworms contained high concentrations of PACAP(-like peptide). Both in developing embryos and mature earthworms occurred the PAC1R(-like peptide) characterised by high affinity to synthetic PACAP38 and its molecular weight was about 50 kDa.

Immunohistochemistry revealed the pattern of both PACAP(-like peptide) and PAC1R(-like peptide) in the ventral nerve cord and peripheral structures (alimentary canal, body wall) of both developing embryos and mature worms.

Based on our result we proposed that PACAP(-like peptide) of earthworm, similarly to vertebrate PACAP, has pleiotropiv function during embryogenesis and later on it acts as neurotransmitter, neuromodulator or neurohormone in mature worms.

By means of mass spectrometry of immunoprecipitated compounds of ventral nerve cord of *E. fetida* and the sequence analysis of isolated compounds six new CAPA-related neuropeptides (SPFPRI/La, APFPRI/La, SPLPRI/La, SFVRI/La, AFVRI/La, SPAFVRI/La) were identified and based on the conservative C-terminal sequences they were named XRI/Lamides. Electron microscopic immunocytochemistry revealed 6 various types of labelled neurons suggesting morphological and functional heterogeneity of CAPA-relative peptides.

Presence and pattern of CAPA-relative peptides expressing neural structures further their differentiation during the embryonic development were also described.

Our results suggest that the phylogenetically conservative CAPA-peptides expressed not only in insects and molluscs but in earthworms, too and they play a role in their neural and neuroendocrin regulation.

PUBLICATIONS

1. Publications related to the to the thesis

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